

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**





(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention  
of the grant of the patent:  
28.01.1998 Bulletin 1998/05

(51) Int Cl.<sup>6</sup> **C07K 14/00, C12N 15/12**

(21) Application number: **90113673.9**

(22) Date of filing: **17.07.1990**

(54) **Tumor necrosis factor (TNF) inhibitor and method for obtaining the same**  
TNF(Tumor Necrosis Factor)-Inhibitor und Verfahren zur Herstellung  
Inhibiteur du facteur de nécrose tumorale (TNF) et méthode d'obtention

(84) Designated Contracting States:  
**AT BE CH DE DK ES FR GB GR IT LI LU NL SE**

(30) Priority: **18.07.1989 US 381080**  
**11.12.1989 US 450329**  
**07.02.1990 US 479661**

(43) Date of publication of application:  
**17.04.1991 Bulletin 1991/16**

(60) Divisional application: **97103361.8**

(73) Proprietor: **AMGEN BOULDER INC.**  
**Boulder, Colorado 80301 (US)**

(72) Inventors:  
• **Brewer, Michael T.**  
**Boulder, Colorado 80302 (US)**  
• **Hale, Karin K.**  
**Boulder, Colorado 80301 (US)**  
• **King, Michael W.**  
**Terre Haute, Indiana 47803 (US)**  
• **Kohn, Tadahiko**  
**Louisville, Colorado 80027 (US)**  
• **Squires, Charles**  
**Boulder, Colorado 80304 (US)**  
• **Thompson, Robert C.**  
**Boulder, Colorado 80303 (US)**

• **Vanderslice, Rebecca W.**  
**Boulder, Colorado 80303 (US)**  
• **Vannice, James**  
**Boulder, Colorado 80303 (US)**

(74) Representative: **Grünecker, Kinkeldey,**  
**Stockmair & Schwanhäusser Anwaltssozietät**  
**Maximilianstrasse 58**  
**80538 München (DE)**

(56) References cited:  
**EP-A- 0 308 378** **DE-A- 3 910 323**

- **JOURNAL OF BIOLOGICAL CHEMISTRY, vol.**  
**265, no. 3, 25 January 1990, BALTIMORE US,**  
**pages 1531-1536; ENGELMANN, H. et al.: "Two**  
**tumor necrosis factor binding proteins purified**  
**from human urine"**
- **JOURNAL OF BIOLOGICAL CHEMISTRY, vol.**  
**264, no. 20, 15 July 1989, BALTIMORE US, pages**  
**1197-1198; ENGELMANN, H. et al.:**
- **JOURNAL OF BIOLOGICAL CHEMISTRY, vol.**  
**264, no. 20, 15 July 1989, BALTIMORE US, pages**  
**1196 - 6-73; SECKINGER, P. et al.: "Purification**  
**and biologic characterization of a specific tumor**  
**necrosis factor alpha inhibitor"**

Remarks:

Divisional application 97103361.8 filed on 28/02/97

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention)

## Description

BACKGROUND OF THE INVENTION

5 Tumor necrosis factors are a class of proteins produced by numerous cell-types, including monocytes and macrophages. At least two TNFs have been previously described, specifically TNF alpha and TNF beta (lymphotoxin).

These known TNFs have important physiological effects on a number of different target cells involved in the inflammatory response. The proteins cause both fibroblasts and synovial cells to secrete latent collagenase and prostaglandin E<sub>2</sub>, and cause osteoblastic cells to carry out bone resorption. These proteins increase the surface adhesive properties of endothelial cells for neutrophils. They also cause endothelial cells to secrete coagulant activity and reduce their ability to lyse clots. In addition they redirect the activity of adipocytes away from the storage of lipids by inhibiting expression of the enzyme lipoprotein lipase. TNFs cause hepatocytes to synthesize a class of proteins known as "acute phase reactants" and they act on the hypothalamus as pyrogens. Through these activities, it has been seen that TNFs play an important part in an organism's response to stress, to infection, and to injury. See, e.g., articles by P.J. Selby et al. in *Lancet*, Feb. 27, 1989, pg. 483; H.F. Starnes, Jr. et al. in *J. Clin. Invest.* 82: 1321 (1988); A. Oliff et al. in *Cell* 50:555 (1987); and A. Waage et al. in *Lancet*, Feb. 14, 1987, pg. 355.

However, despite their normally beneficial effects, circumstances have come to light in which the actions of TNFs are harmful. For example, TNF alpha injected into animals gives rise to the symptoms of septic shock; endogenous TNF levels have been observed to increase following injection of bacteria or bacterial cell walls. TNFs also cause bowel necrosis and acute lung injury, and they stimulate the catabolism of muscle protein. In addition, the ability of TNFs to increase the level of collagenase in an arthritic joint and to direct the chemotaxis and migration of leukocytes and lymphocytes may also be responsible for the degradation of cartilage and the proliferation of the synovial tissue in this disease. Therefore, TNFs may serve as mediators of both the acute and chronic stages of immunopathology in rheumatoid arthritis. TNFs may also be responsible for some disorders of blood clotting through altering endothelial cell function. Moreover, excessive TNF production has been demonstrated in patients with AIDS and may be responsible for some of the fever, acute phase response and cachexia seen with this disease and with leukemias.

In these and other circumstances in which TNF has a harmful effect, there is clearly a clinical use for an inhibitor of TNF action. Systemically administered, TNF inhibitors would be useful therapeutics against septic shock and cachexia. Locally applied, such TNF inhibitors would serve to prevent tissue destruction in an inflamed joint and other sites of inflammation. Indeed, such TNF inhibitors could be even more effective when administered in conjunction with interleukin-1 (IL-1) inhibitors.

One possibility for therapeutic intervention against the action of TNF is at the level of the target cell's response to the protein. TNF appears to act on cells through a classical receptor-mediated pathway. Thus, any molecule which interferes with the ability of TNF to bind to its receptors either by blocking the receptor or by blocking the TNF would regulate TNF action. For these reasons, proteins and small molecules capable of inhibiting TNF in this manner have been sought.

In EP-A-308378, a TNF inhibitory protein originating from human urine and behaving as having an apparent molecular weight of 40 to 80 kDa on an Ultrogel ACA 44 gel filtration column is disclosed. When analysed on SDS PAGE under reducing conditions, the protein has a molecular weight of 26 to 28 kDa. The N-terminus of the protein was determined to have the following amino acid sequence: Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser.

A protein having the N-terminal amino acid sequence Asp-Ser-Val-Cys-Pro was also disclosed by Engelmann et al., *J. Biol. Chem.* 264, 1989, pp 11974-11980. The protein was shown to be a TNF inhibitor having an apparent molecular weight of 27,000 kDa on SDS-PAGE under reducing and non-reducing conditions. The protein interfered with the binding of TNF alpha and TNF beta. Specific binding of the protein to TNF alpha and to a lesser extent to TNF beta was observed.

Seckinger et al., *J. Biol. Chem.* 264, 1989, pp 11966-11973, disclose a TNF alpha inhibitor migrating with an apparent molecular weight of 43,000 kDa under reducing and non-reducing conditions. No amino acid sequence is provided. Further, DE-A-3910323 discloses a TNF alpha inhibitor having a molecular weight of 40 to 60 kDa when determined by molecular sieve chromatography and of 33 kDa as determined by SDS-PAGE. The N-terminal amino acid sequence was determined to be Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-Cys-Asn-Ser-Ile.

In a further publication, Engelmann et al. (*J. Biol. Chem.* 265, 1990, pp 1531-1536) disclose two TNF inhibitors both having a molecular weight of about 30,000 kDa. The N-terminal sequence of these proteins are determined to be (i) Asp-Ser-Val-Cys-Pro and (ii) Val-Ala-Phe-Thr-Pro, with sequence (i) being invariable and sequence (ii) being truncated to varying degrees to result in proteins having the N-terminal sequences Thr-Pro and Phe-Thr-Pro.

SUMMARY OF THE INVENTION

An object of the present invention is to provide purified forms of TNF inhibitor which are active against TNF alpha to enable the determination of their amino acid sequence. An additional object is to provide further TNF inhibitory molecules.

A further object is to provide the amino acid sequences of certain TNF inhibitors. In addition it is an object of this invention to provide a cellular source of the mRNA coding for TNF inhibitors and a cDNA library containing a cDNA for the inhibitors. Furthermore, it is an object of this invention to provide a genomic clone of DNA coding for the TNF inhibitors, and the coding sequences of that DNA.

The identification of biologically-active analogs of such TNF inhibitors with enhanced or equivalent properties is also one of the objects of the invention.

Additionally, it is an object of this invention to provide a recombinant-DNA system for the production of the TNF inhibitor described herein. A further object of the present invention includes providing purified forms of TNF inhibitor which would be valuable as pharmaceutical preparations exhibiting activity against TNF. Another object of the present invention includes providing purified combinations of TNF inhibitors and IL-1 inhibitors which are valuable as pharmaceutical preparations exhibiting activity against both IL-1 and TNF.

The inventors of the present invention have isolated at least two TNF inhibitor proteins with TNF-inhibiting properties. A 30kDa protein and a 40kDa protein have been obtained in their purified forms. The amino acid sequence of the 30kDa TNF inhibitor protein has been obtained. The amino acid sequence data of the 40kDa TNF inhibitor protein has also been obtained. The 40kDa TNF inhibitor is a novel, previously undescribed protein.

A human genomic DNA clone which contains the gene for the 30kDa protein has been obtained. A cell source of this protein has been identified and a cDNA clone has been obtained and the nucleic acid sequence of the gene for the protein determined. In addition, the gene clone has been placed in a vector which has been found to express the protein in host cells. Also a process has been developed for purifying the protein in an active form.

A cell source has been identified which produces the 40kDa protein and a cDNA clone has been obtained and the nucleic acid sequence determined of the gene for the 40kDa protein. The full cDNA clones encoding for both the 30kDa TNF inhibitor precursor and the 40kDa TNF inhibitor precursor have been expressed in mammalian cells to yield an increase in TNF binding sites on the cell surface.

A gene coding for the mature form of the 30kDa protein has been expressed in E. Coli. Three separate genes coding for all or portions of the mature 40kDa protein have also been expressed in E. Coli. The three 40kDa inhibitor proteins expressed-- mature 40kDa TNF inhibitor, 40kDa TNF inhibitor Δ51 and 40kDa TNF inhibitor Δ53--each exhibit TNF inhibiting activity. Mature 40kDa TNF inhibitor, as isolated from medium conditioned by human U937 cells, and 40kDa TNF inhibitor Δ 51 and 40kDa TNF inhibitor Δ53, are collectively referred to as 40kDa TNF inhibitor.

The 30kDa TNF inhibitor has shown activity in inhibiting TNF alpha. The 40kDa TNF inhibitor has shown inhibitory action against both TNF alpha and TNF beta.

It will now be possible to perform the large scale production of these TNF inhibitors through recombinant DNA technology. These inhibitors should be suitable for use in pharmaceutical formulations useful in treating pathophysiological conditions mediated by TNF.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 describes a cytotoxicity assay for TNF in the absence (- -) and in the presence (-x-x-) of TNF inhibitor (30kDa). Various concentrations of TNF were incubated with and without TNF inhibitor (30kDa), and the cytotoxicity assay was performed as described in Example 1.

Figure 2 describes a native gel shift assay in which "a" depicts TNF alone, and "b" depicts TNF + TNF inhibitor (30kDa).

Figure 3 describes Con A-Peroxidase staining of TNF inhibitor (30kDa). About 200 ng of each protein were run on 14% SDS-PAGE, and transferred to nitrocellulose filter. Glycoproteins were identified using Con A-peroxidase staining. In this figure, "a" depicts a molecular weight marker, "b" depicts Ovalbumin, "c" depicts bovine serum albumin and "d" depicts TNF inhibitor (30kDa).

Figure 4 describes chemical deglycosylation of TNF inhibitor (30kDa). About 200 ng of TNF inhibitor (30kDa) were chemically deglycosylated (lane C) as described in Example 1.

Figure 5 describes N-glycanase treatment of TNF inhibitor (30kDa). Purified TNF inhibitor (30kDa) was iodinated by Bolton-Hunter reagent, and denatured-iodinated TNF inhibitor (30kDa) was treated with N-glycanase for 6 hours at 37C. In this figure, "a" depicts native TNF inhibitor (20kDa) and "b" depicts deglycosylated TNF inhibitor (30kDa).

Figure 6A describes an OD<sub>280</sub> profile of the DEAE Sepharose CL-6B chromatography of 20 1 urine.

Figure 6B describes an autoradiograph of the corresponding native gel shift assay indicating a peak of TNF inhibitor (30kDa) at fraction 57-63, which is about 20mM NaCl.

Figure 7 describes an OD<sub>230</sub> profile of the 0.05 M Na Phosphate pH 2.5 elution from the TNF affinity column.

Figure 8A and 8C describe chromatographic profiles (OD<sub>215</sub> and OD<sub>230</sub>) of the RP8 purification of the TNF inhibitor (30kDa) with the L929 bioassay of fractions from the RP8 column showing a peak of TNF inhibitor (30kDa) at fractions 25-31 which is about 12% acetonitrile and at fractions 35 and 36 which is about 21% acetonitrile.

Figure 8B describes a silver stained 15% SDS-PAGE of the RP8 pool showing a single band at 30kDa.

Figure 9A describes a peptide purification of Lys-C digestion of TNF inhibitor (30kDa).

Figure 9B describes a peptide purification of alkylated ( ) Lys-C digests of TNF inhibitor (30kDa).

Figure 10 describes a peptide purification of two alkylated ( ) Asp-N digests of TNF inhibitor (30kDa).

Figures 11A and 11B describe peptide purifications of an endopeptidase V8 digest of reduced carboxymethylated TNF inhibitor (30kDa).

Figure 12 describes amino acid sequences present in TNF inhibitor (30kDa). Blanks in the sequence indicate the residue has not been unambiguously identified by protein sequencing. C- indicates the identification of carboxymethylcysteine by the presence of <sup>3</sup>H in the residue.

Figure 13 describes the DNA sequence of a genomic clone encoding at least a portion of a TNF inhibitor (30kDa).

Figure 14 describes at least 70% of the mature amino acid sequence of a preferred TNF inhibitor.

Figure 15 describes detection of TNF inhibitor in U937 supernatant by the gel shift assay.

Figure 16 describes detection of TNF inhibitor in hplc fractions from U937 supernatant.

Figure 17 describes the Northern blot according to Example 4.

Figure 18 describes the deglycosylated TNF inhibitor (30kDa) binding to TNF. Glycosylated and deglycosylated TNF inhibitor were incubated with TNF affigel, and flow through materials and eluates of the gel were analysed on SDS-PAGE. In this figure, (11) indicated flow through of TNF-INH, reduced and oxidized, (21) indicated flow through of deglycosylated TNF-INH, reduced and oxidized, (51) indicates flow through of native TNF-INH, (12) indicates eluate of TNF-INH, reduced and oxidized, (22) indicates eluate of deglycosylated TNF-INH, reduced and oxidized, and (52) indicates eluate of native TNF-INH.

Figure 19 describes the complete amino acid sequence of the 30kDa TNF inhibitor.

Figure 20 describes the cDNA sequence encoding the amino acid sequence shown in Figure 19.

Figure 21 describes the entire cDNA sequence for the precursor of the 30kDa TNF inhibitor.

Figure 22 describes the DNA sequence near the start of the TNF inhibitor (30kDa) gene in plasmid PTNFIX-1.

Figure 23 describes the plasmid pCMVXV beta TNFBP stop A.

Figure 24 describes the plasmid pSVXVTNFBP stop A.

Figure 25 describes a chromatographic profile OD<sub>215</sub> of the RP8 column of the 30kDa TNF inhibitor from E. Coli. The L929 bioassay results are also shown (-x-x).

Figure 26 describes a silver stained 14% SDS-PAGE of the RP8 Fractions in Figure 25.

Figure 27 describes a chromatographic profile OD<sub>215</sub> of the RP8 purification of the TNF inhibitors from U937 cells.

The L929 bioassay results are also shown with a bar graph. Two distinctive TNF inhibitor peaks are seen.

Figure 28 describes a silver stained 14% SDS-PAGE of the RP8 fractions. Fraction number 30 contains the 30kDa TNF inhibitor and fraction number 35 contains the 40kDa TNF inhibitor.

Figure 29 describes a chromatographic profile OD<sub>215</sub> of the purification of urinary 40kDa TNF inhibitor. The second TNF inhibitory peak from several RP8 chromatographies were combined and reanalyzed on an RP8 column. TNF-inhibitory activity is shown with a bar graph. The difference between the OD<sub>215</sub> peak and the activity peak reflects the dead volume between the detector and the fraction collector.

Figure 30 describes a silver stained 14% SDS-PAGE of the RP8 fractions of urine. Fraction number 32 contains the 40kDa TNF inhibitor.

Figure 31 describes the amino terminal sequences of U937 derived inhibitors (30kDa and 40kDa), and urine-derived 40kDa TNF inhibitor.

Figure 32 describes a peptide purification of endopeptidase V8 digested 40kDa TNF inhibitor.

Figure 33 describes a peptide purification endopeptidase Arg-C digested 40kDa TNF inhibitor.

Figure 34 describes a peptide purification of trypsin digested Arg-C16 peptide.

Figure 35 describes a peptide purification of chymotrypsin digested Arg-C10 peptide.

Figure 36 describes a primary structure of the 40kDa TNF inhibitor.

Figure 37 describes a portion of the 40kDa TNF inhibitor cDNA sequence along with the predicted amino acid translation product.

Figure 38 describes the complete amino acid sequence of the 40kDa TNF inhibitor.

Figure 39 describes the entire cDNA sequence for the precursor of the 40kDa TNF inhibitor along with its deduced translation product.

Figure 40 describes a cytotoxicity assay for TNF beta (lymphotoxin) in the presence (o-o) of 40kDa TNF inhibitor in the presence (●-●) of 30kDa TNF inhibitor and without any inhibitor (x-x).

Figure 41 describes the expression of the 30kDa TNF inhibitor cDNA sequence shown in Figure 21 in CoS7 cells.

COS cells were transfected with plasmids using the lipofectin procedure of Feigner *et al.* (Proc. Natl. Acad. Sci. (USA) 84: 7413-1987).  $3.4 \times 10^5$  cells were incubated with the indicated amounts of [ $^{125}$ I] TNF $\alpha$  at a specific activity of  $5.6 \times 10^4$  cpm/mg and the amount bound to the cells determined. Open symbols are the total cpm associated with cells after a 4 hour incubation at 4°C. Closed symbols represent bound [ $^{125}$ I] TNF $\alpha$  in the presence of 130 fold excess of cold unlabeled TNF $\alpha$ .

Figure 42 describes the expression of the 40kDa TNF inhibitor cDNA sequence shown in Figure 39 in COS7. Assay conditions were as described in Figure 41. The darkened symbols represent the bound [ $^{125}$ I] TNF $\alpha$  in the presence of 130 fold excess of cold unlabeled TNF $\alpha$ .

Figure 43 describes the cytotoxicity assay of an HPLC RPC-8 fraction of the human monocytes which were treated with PMA and PHA for 24 hours.

Figure 44 describes the RPC-8 chromatographic pattern of 40kDa TNF inhibitor  $\Delta 51$  (A), SDS-polyacrylamide gel analysis of the fractions (B), and the cytotoxicity assay on L929 cells (C).

Figure 45 describes the RPC-8 chromatographic pattern of 40kDa TNF inhibitor  $\Delta 53$  (A), SDS-polyacrylamide gel analysis of the fractions (B), and the cytotoxicity assay on L929 cells (C).

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principals of the invention.

### 1. Inhibitor isolated from urine.

As noted above, the present invention relates to TNF inhibitors which have been isolated in a purified form. In one embodiment of this invention, the 40kDa TNF inhibitor is preferably derived from urine. In addition, the invention encompasses substantially purified TNF inhibitors of any origin which are biologically equivalent to the inhibitor isolated from urine. Throughout this specification, any reference to a TNF inhibitor or simply an inhibitor should be construed to refer to each of the inhibitors identified and described herein.

By "biologically equivalent" as used throughout the specification and claims, we mean compositions of the present invention which are capable of preventing TNF action in a similar fashion, but not necessarily to the same degree as the native TNF inhibitor isolated from urine. By "substantially homologous" as used throughout the ensuing specification and claims, is meant a degree of homology to the native TNF inhibitor isolated from urine in excess of that displayed by any previously reported TNF inhibitor. Preferably, the degree of homology is in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90%, 95% or 99%. A particularly preferred group of TNF inhibitors are in excess of 95% homologous with the native inhibitor. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment as set forth by Dayhoff, in Atlas of Protein Sequence and Structure Vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by reference. Also included as substantially homologous are those TNF inhibitors which may be isolated by virtue of cross-reactivity with antibodies to the described inhibitor or whose genes may be isolated through hybridization with the gene or with segments of the described inhibitor.

The preferred 40kDa TNF inhibitor of the present invention has been derived from urine and, for the first time, has been isolated in a purified form.

For the purposes of the present application, "pure form" or "purified form," when used to refer to the TNF inhibitor disclosed herein, shall mean a preparation which is substantially free of other proteins which are not TNF inhibitor proteins. Preferably, the TNF inhibitor of the present invention is at least 50% percent pure, preferably 75% pure and more preferably 80%, 95% or 99% pure. In one embodiment of the present invention, the TNF inhibitor protein preparation is sufficiently pure to enable one of ordinary skill in the art to determine its amino acid sequence without first performing further purification steps.

At least two TNF inhibitors have been isolated by the methods set forth in the examples. The two inhibitors are approximately 30kDa and approximately 40kDa molecules on SDS-PAGE. The 30kDa inhibitor eluates from a DEAE CL6B column at about 20 millimolar NaCl in Tris buffer, pH 7.5. The amino acid sequence of the 30kDa inhibitor is set forth in Figure 19, and the amino acid sequence of the 40kDa inhibitor is set forth in Figure 38. The 30kDa TNF inhibitor has been shown to inhibit the activity of TNF alpha, and has little effect on the activity of TNF beta. The 40kDa TNF inhibitor has been shown to exhibit a significant inhibiting effect against both TNF alpha and TNF beta (lymphotoxin).

## 2 Inhibitor isolated from U937 condition medium

In an alternate embodiment of the present invention the 40kDa TNF inhibitor is isolated from a medium conditioned by human U937 cells. The 40kDa protein isolated from U937 conditioned medium is substantially homologous to the 40kDa TNF inhibitor isolated from urine and is biologically equivalent to said protein.

## 3 Structure of 30kDa TNF inhibitor

The 30kDa TNF inhibitor isolatable from urine is a glycoprotein, containing at least one carbohydrate moiety. In one embodiment of this invention, the natural 30kDa TNF inhibitor is deglycosylated. The deglycosylated TNF inhibitor, which retains its ability to bind to TNF, is within the scope of the present invention. Fully and partially deglycosylated 30kDa TNF inhibitor is encompassed by this invention. The deglycosylated 30kDa TNF inhibitor isolated from urine is about an 18kDa protein.

The gene sequence identified that encodes the 30kDa protein does not contain a termination codon as would be anticipated for the amino acid sequence of the 18kDa protein. The inventors theorize, but are in no way to be limited by this theory, that the proteins produced in vivo contain additional amino acid sequences. According to this theory, the protein encoded is a TNF receptor molecule. The inhibitor protein encoded by the cDNA has a hydrophobic sequence that would be compatible with the cell membrane spanning region and a TNF binding portion that would extend extracellularly from the cell membrane. In accord with this hypothesis and as described in Example 19, the cDNA has been expressed in COS cells and leads to an increase in the number of TNF binding sites on the cell. The TNF inhibitors of the present invention, therefore, are the receptor fragments or portions of the receptor molecule. Such binding fragments have been identified with respect to other binding/inhibitory molecules (e.g., IL-2 inhibitor), and are referred to as soluble receptors.

This theory is consistent with the lack of a termination codon in the nucleotide sequence that would correspond to the terminus of the protein as anticipated by the known sequence of the isolated TNF inhibitor factor. It is also consistent with the fact that the nucleotide sequence beyond where the termination codon should be found, encodes a series of hydrophobic amino acids.

The present invention, therefore, encompasses not just the portion of the TNF inhibitors identified and described, but all proteins containing any portion of the amino acid sequence encoded by the cDNA sequences identified and described herein.

## 4. Structure of 40kDa TNF inhibitor

The 40kDa TNF inhibitor isolated from medium conditioned by human U937 cells and identified in urine is a glycoprotein, containing at least one carbohydrate moiety. In one embodiment of this invention, the natural 40kDa TNF inhibitor is deglycosylated. The deglycosylated 40kDa TNF inhibitor, which retains its ability to bind to both TNF alpha and TNF beta (lymphotoxin) is within the scope of the present invention. Fully and partially deglycosylated 40kDa TNF inhibitor is encompassed by this invention. The inventors theorize that the 40kDa TNF inhibitor may also be a soluble receptor. The gene sequence identified that encodes the 40kDa protein does not contain a termination codon as would be anticipated for the amino acid sequence of the deglycosylated 40kDa TNF inhibitor. As described in Example 20, the cDNA has been expressed in COS cells and leads to an increase in the number of TNF binding sites on the cell.

The present invention encompasses the gene encoding the mature 40kDa protein isolated from medium conditioned by human U937 cells and identified in urine, and larger and smaller portions of such gene to the extent that the TNF inhibiting activity of the encoded protein is not effected. As can be seen by reference to Figure 38, the mature 40kDa TNF inhibitor has a proline rich area near the anticipated C-terminus of the protein. 40kDa TNF inhibitors in which all or portions of the proline rich region are not included in the protein are active as TNF inhibitors, and are within the scope of the present invention. Two such shortened proteins are described in Examples 17 and 22 below, and are referred to as 40kDa TNF inhibitor Δ51 and 40kDa TNF inhibitor Δ53. All portions of this application which refer generally to 40kDa TNF inhibitor shall encompass the mature 40kDa protein isolated from medium conditioned by human U937 cells and identified in urine, as well as 40kDa TNF inhibitor Δ51 and 40kDa TNF inhibitor Δ53.

It is generally believed that at least one TNF receptor is capable of binding both TNF alpha and TNF beta, while some TNF receptors are capable of only binding TNF alpha. This is consistent with the findings that there are two TNF inhibitors which are both proposed to be active fragments of TNF receptor sites, and one is active against only TNF alpha and the other is active against both TNF alpha and TNF beta.



## 5 Recombinant inhibitor

### (a) General

5 A recombinant DNA method for the manufacture of a TNF inhibitor is now disclosed. In one embodiment of the invention, the active site functions in a manner biologically equivalent to that of the TNF inhibitor isolated from urine. A natural or synthetic DNA sequence may be used to direct production of such TNF inhibitors. This method comprises

- 10 (a) preparation of a DNA sequence capable of directing a host cell to produce a protein having TNF inhibitor activities or a precursor thereof;
- (b) cloning the DNA sequence into a vector capable of being transferred into and replicated in a host cell, such vector containing operational elements needed to express the DNA sequence or a precursor thereof;
- (c) transferring the vector containing the synthetic DNA sequence and operational elements into a host cell capable of expressing the DNA encoding TNF inhibitor or a precursor thereof;
- 15 (d) culturing the host cells under the conditions for amplification of the vector and expression of the inhibitor or a precursor thereof;
- (e) harvesting the inhibitor or a precursor thereof; and
- (f) permitting the inhibitor to assume an active tertiary structure whereby it possesses or can be processed into a protein having TNF inhibitory activity.

20 In one embodiment of the present invention, the TNF inhibitor is produced by the host cell in the form of a precursor protein. This precursor protein is processed to a protein in one or more steps and allowed to fold correctly to an active TNF inhibitor using methods generally known to those of ordinary skill in the art.

### 25 (b) DNA sequences

DNA sequences contemplated for use in this method are discussed in part in Examples 6, 14A, and 17. It is contemplated that these sequences include synthetic and natural DNA sequences and combinations thereof. The natural sequences further include cDNA or genomic DNA segments.

- 30 The means for synthetic creation of polynucleotide sequences encoding a protein identical to that encoded by the cDNA or genomic polynucleotide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As an example of the current state of the art relating to polynucleotide synthesis, one is directed to Matteucci, M.D., and Caruthers, M.H., in J. Am. Chem. Soc. 103:3185 (1981) and Beaucage, S.L. and Caruthers, M.H. in Tetrahedron Lett. 22:1859 (1981), and to the instructions supplied with an ABI oligonucleotide synthesizer, each of which is specifically incorporated herein by reference.

- 35 These synthetic sequences may be identical to the natural sequences described in more detail below or they may contain different nucleotides. In one embodiment, if the synthetic sequences contain nucleotides different from those found in the natural DNA sequences of this invention, it is contemplated that these different sequences will still encode a polypeptide which has the same primary structure as TNF inhibitor isolated from urine. In an alternate embodiment, the synthetic sequence containing different nucleotides will encode a polypeptide which has the same biological activity as the TNF inhibitor described herein.

40 Additionally, the DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature and which has been isolated and purified for the first time by the present inventors. In one embodiment, the DNA sequence is a restriction fragment isolated from a cDNA library.

- 45 In an alternative embodiment, the DNA sequence is isolated from a human genomic library. An example of such a library useful in this embodiment is set forth by Wyman, et al., (1985) Proc. Nat. Acad. Sci. USA. 82: 2880-2884.

In a preferred version of this embodiment, it is contemplated that the natural DNA sequence will be obtained by a method comprising:

- 50 (a) Preparation of a human cDNA library from cells, preferably U937 cells capable of generating a TNF inhibitor, in a vector and a cell capable of amplifying and expressing all or part of that cDNA;
- (b) Probing the human DNA library with at least one probe capable of binding to the TNF inhibitor gene or its protein product;
- (c) Identifying at least one clone containing the gene coding for the inhibitor by virtue of the ability of the clone to bind at least one probe for the gene or its protein product;
- 55 (d) Isolation of the gene or portion of the gene coding for the inhibitor from the clone or clones chosen; and
- (e) Linking the gene, or suitable fragments thereof, to operational elements necessary to maintain and express the gene in a host cell.

The natural DNA sequences useful in the foregoing process may also be identified and isolated through a method comprising:

- (a) Preparation of a human genomic library preferably propagated in a recBc sbc host preferably CES 200
- (b) Probing the human genomic library with at least one probe capable of binding a TNF inhibitor gene or its protein product;
- (c) Identification of at least one clone containing the gene coding for the inhibitor by virtue of the ability of the clone to bind at least one probe for the gene or its protein product;
- (d) Isolation of the gene coding for the inhibitor from the clone or clones identified; and
- (e) Linking the gene, or suitable fragments thereof, to operational elements to maintain and express the gene in a host cell.

A third potential method for identifying and isolating natural DNA sequences useful in the foregoing process includes the following steps:

- (a) Preparation of mRNA from cells that produce the TNF inhibitor;
- (b) Synthesizing cDNA (single- or double-stranded) from this mRNA;
- (c) Amplifying the TNF inhibitor-specific DNA sequences present in this mixture of cDNA sequences using the polymerase chain reaction (PCR) procedure with primers such as those shown in Table 5.
- (d) Identifying the PCR products that contain sequences present in the other oligonucleotide probes shown in Table 5 using Southern blotting analysis;
- (e) Subcloning the DNA fragments so identified into M13 vectors that allow direct sequencing of the DNA sequences;
- (f) Using these sequences to isolate a cDNA clone from a cDNA library; and
- (g) Linking the gene, or suitable fragments thereof, to operational elements necessary to maintain and express the gene in host cells.

In isolating a DNA sequence suitable for use in the above-method, it is preferred to identify the two restriction sites located within and closest to the end portions of the appropriate gene or sections of the gene that encode the native protein or fragments thereof. The DNA segment containing the appropriate gene or sections of the gene is then removed from the remainder of the genomic material using appropriate restriction endonucleases. After excision, the 3' and 5' ends of the DNA sequence and any intron exon junctions are reconstructed to provide appropriate DNA sequences capable of coding for the N- and C- termini and the body of the TNF inhibitor protein and capable of fusing the DNA sequence to its operational elements.

As described in Example 17 below, the DNA sequence utilized for the expression of 40kDa TNF inhibitor may be modified by the removal of either 153 or 159 base pairs from the gene that encodes for the mature 40kDa TNF inhibitor isolated from medium conditioned by human U937 cells and identified in urine. The  $\Delta 53$  gene was prepared to remove the proline region from the C-terminus of the full gene, and the  $\Delta 51$  gene was prepared to approximate the C-terminus of the gene encoding for 30kDa TNF inhibitor.

A DNA sequence, isolated according to these methods from a cDNA library and encoding at least a portion of the 30kDa TNF inhibitor described herein, has been deposited at the American Type Culture Collection, Rockville, MD, under Accession No 40645.

A DNA sequence, isolated according to these methods from a human genomic DNA library and encoding at least a portion of the 30kDa TNF inhibitor described herein, has been deposited at the American Type Culture Collection, Rockville, MD, under Accession No. 40620.

A DNA sequence, isolated according to these methods from a cDNA library and encoding at least a portion of the 40kDa TNF inhibitor described herein, has been deposited at the American Type Culture Collection, Rockville, MD, under Accession No. 62204.

## 6 Vectors

### (b) Microorganisms, especially *E. coli*

The vectors contemplated for use in the present invention include any vectors into which a DNA sequence as discussed above can be inserted, along with any preferred or required operational elements, and which vector can then be subsequently transferred into a host cell and replicated in such cell. Preferred vectors are those whose restriction sites have been well documented and which contain the operational elements preferred or required for transcription of the DNA sequence. However, certain embodiments of the present invention are also envisioned which employ cur-

reptly undiscovered vectors which would contain one or more of the cDNA sequences described herein. In particular it is preferred that all of these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stably maintained and propagated in the desired host; (3) be capable of being present in a high copy number in the desired host; (4) possess a regulatable promoter positioned so as to promote transcription of the gene of interest; (5) have at least one marker DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the DNA sequence will be inserted; and (6) a DNA sequence capable of terminating transcription.

In variously preferred embodiments, these cloning vectors containing and capable of expressing the DNA sequences of the present invention contain various operational elements. These "operational elements," as discussed herein, include at least one promoter, at least one Shine-Dalgarno sequence and initiator codon, and at least one terminator codon. Preferably, these "operational elements" also include at least one operator, at least one leader sequence for proteins to be exported from intracellular space, at least one gene for a regulator protein, and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA.

Certain of these operational elements may be present in each of the preferred vectors of the present invention. It is contemplated that any additional operational elements which may be required may be added to these vector using methods known to those of ordinary skill in the art, particularly in light of the teachings herein.

In practice, it is possible to construct each of these vectors in a way that allows them to be easily isolated, assembled and interchanged. This facilitates assembly of numerous functional genes from combinations of these elements and the coding region of the DNA sequences. Further, many of these elements will be applicable in more than one host. It is additionally contemplated that the vectors, in certain preferred embodiments, will contain DNA sequences capable of functioning as regulators ("operators"), and other DNA sequences capable of coding for regulator proteins.

#### (i) Regulators

These regulators, in one embodiment, will serve to prevent expression of the DNA sequence in the presence of certain environmental conditions and, in the presence of other environmental conditions, will allow transcription and subsequent expression of the protein coded for by the DNA sequence. In particular, it is preferred that regulatory segments be inserted into the vector such that expression of the DNA sequence will not occur, or will occur to a greatly reduced extent, in the absence of, for example, isopropylthio-beta-D-galactoside. In this situation, the transformed microorganisms containing the DNA sequence may be grown to a desired density prior to initiation of the expression of TNF inhibitor. In this embodiment, expression of the desired protein is induced by addition of a substance to the microbial environment capable of causing expression of the DNA sequence after the desired density has been achieved.

#### (ii) Promoters

The expression vectors must contain promoters which can be used by the host organism for expression of its own proteins. While the lactose promoter system is commonly used, other microbial promoters have been isolated and characterized, enabling one skilled in the art to use them for expression of the recombinant TNF inhibitor.

#### (iii) Transcription Terminator

The transcription terminators contemplated herein serve to stabilize the vector. In particular, those sequences as described by Rosenberg, M. and Court, D., in *Ann. Rev. Genet.* 13:319-353 (1979), specifically incorporated herein by reference, are contemplated for use in the present invention.

#### (iv) Non-Translated Sequence

It is noted that, in the preferred embodiment, it may also be desirable to reconstruct the 3' or 5' end of the coding region to allow incorporation of 3' or 5' non-translated sequences into the gene transcript. Included among these non-translated sequences are those which stabilize the mRNA as they are identified by Schmeissner, U., McKenney, K., Rosenberg, M. and Court, D., in *J. Mol. Biol.* 176:39-53 (1984), specifically incorporated herein by reference.

#### (v) Ribosome Binding Sites

The microbial expression of foreign proteins requires certain operational elements which include, but are not limited to, ribosome binding sites. A ribosome binding site is a sequence which a ribosome recognizes and binds to in the initiation of protein synthesis as set forth in Gold, L., *et al.*, *Ann. Rev. Microbio.* 35:557-580; or Marquis, D.M., *et al.*, *Gene* 42:175-183 (1986), both of which are specifically incorporated herein by reference. A preferred ribosome binding

Site is GAGGCGCAAAA(ATG)

(vi) Leader Sequence and Translational Coupler

5 Additionally, it is preferred that DNA coding for an appropriate secretory leader (signal) sequence be present at the 5' end of the DNA sequence as set forth by Watson, M.E. in *Nucleic Acids Res.* 12:5145-5163, specifically incorporated herein by reference, if the protein is to be excreted from the cytoplasm. The DNA for the leader sequence must be in a position which allows the production of a fusion protein in which the leader sequence is immediately adjacent to and covalently joined to the inhibitor, i.e., there must be no transcription or translation termination signals between the two DNA coding sequences. The presence of the leader sequence is desired in part for one or more of the following reasons. First, the presence of the leader sequence may facilitate host processing of the TNF inhibitor. In particular, the leader sequence may direct cleavage of the initial translation product by a leader peptidase to remove the leader sequence and leave a polypeptide with the amino acid sequence which has potential protein activity. Second, the presence of the leader sequence may facilitate purification of the TNF inhibitor, through directing the protein out of the cell cytoplasm. In some species of host microorganisms, the presence of an appropriate leader sequence will allow transport of the completed protein into the periplasmic space, as in the case of some *E. coli*. In the case of certain *E. coli*, *Saccharomyces* and strains of *Bacillus* and *Pseudomonas*, the appropriate leader sequence will allow transport of the protein through the cell membrane and into the extracellular medium. In this situation, the protein may be purified from extracellular protein. Thirdly, in the case of some of the proteins prepared by the present invention, the presence of the leader sequence may be necessary to locate the completed protein in an environment where it may fold to assume its active structure, which structure possesses the appropriate protein activity.

In one preferred embodiment of the present invention, an additional DNA sequence is located immediately preceding the DNA sequence which codes for the TNF inhibitor. The additional DNA sequence is capable of functioning as a translational coupler, i.e., it is a DNA sequence that encodes an RNA which serves to position ribosomes immediately adjacent to the ribosome binding site of the inhibitor RNA with which it is contiguous. In one embodiment of the present invention, the translational coupler may be derived using the DNA sequence

30 **TAACGAGGCGCAAAAAATGAAAAGACAGCTATCGCGATCTTGGAGGATGATTAAATC**

and methods currently known to those of ordinary skill in the art related to translational couplers.

35 (vii) Translation Terminator

The translation terminators contemplated herein serve to stop the translation of mRNA. They may be either natural, as described by Kohli, J., *Mol. Gen. Genet.* 192:430-439, or synthesized, as described by Pettersson, R.F. *Gene* 24:15-27 (1983), both of which references are specifically incorporated herein by reference.

40 (viii) Selectable Marker

45 Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host microorganism. In one embodiment of the present invention, the gene for ampicillin resistance is included in the vector while, in other plasmids, the gene for tetracycline resistance or the gene for chloramphenicol resistance is included.

Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants. Additionally, the presence of such a selectable marker in the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, a pure culture of the transformed host microorganisms would be obtained by culturing the microorganisms under conditions which require the induced phenotype for survival.

50 The operational elements as discussed herein are routinely selected by those of ordinary skill in the art in light of prior literature and the teachings contained herein. General examples of these operational elements are set forth in B. Lewin, *Genes*, Wiley & Sons, New York (1983), which is specifically incorporated herein by reference. Various examples of suitable operational elements may be found on the vectors discussed above and may be elucidated through review of the publications discussing the basic characteristics of the aforementioned vectors.

55 Upon synthesis and isolation of all necessary and desired component parts of the above-discussed vector, the vector is assembled by methods generally known to those of ordinary skill in the art. Assembly of such vectors is

believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, is capable of being performed without undue experimentation. For example, similar DNA sequences have been ligated into appropriate cloning vectors, as set forth by Maniatis et al. in Molecular Cloning, Cold Spring Harbor Laboratories (1984) which is specifically incorporated herein by reference.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the DNA sequence and its attendant operational elements may be inserted into each vector. In such an embodiment the host organism would produce greater amounts per vector of the desired TNF inhibitor. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and transcribed in an appropriate host cell.

#### (b) Other Microorganisms

Vectors suitable for use in microorganisms other E. coli are also contemplated for this invention. Such vectors are described in Table 1. In addition, certain preferred vectors are discussed below.

TABLE 1

| HOSTS               | REGULATED PROMOTERS  | INDUCER  | TRANSCRIPTION TERMINATION  | NRMA STABILIZATION  | TRANSCRIPTIONAL START SITE & LEADER PEPTIDE   | MARKER  | RS BINDING SITE               |
|---------------------|--|--|--|---|---|---|-------------------------------|
| <i>E. coli</i>      | $\text{lac}^1$ , $\text{lac}^2$<br>$\text{lam}^3$ , $\text{pl}^4$<br>$\text{trp}^5$  | IP1G<br>increased temperature<br>IAA addition<br>or tryptophan depletion       | $\text{rrn}^6$<br>$\text{rrn}^7$                                 | $\text{omp}^8$<br>$\text{lam}^9$ , $\text{int}^9$<br>$\text{trp}^9$ | $\text{bla}^{11}$<br>$\text{omp}^8$<br>$\text{pho}^8$   | $\text{amp}^{\text{icilin}}^{14}$<br>tetracycline <sup>14,15</sup><br>chloramphenicol <sup>16</sup> |                               |
| <i>Bacillus</i>     | $\alpha$ -amylase <sup>17</sup><br>$\beta$ -subtilisin <sup>18</sup><br>$\alpha$ -D <sup>43</sup><br>$\text{spe}^{\text{m}}^{126}$ | IP1G   | <i>E. coli</i> $\text{rrn}^6$<br>$\text{rrn}^7$ , $\text{trp}^9$ |   | <i>B. amy</i> neutral protease <sup>24</sup><br><i>B. amy</i> $\alpha$ -amylase <sup>22</sup><br><i>B. subtilis</i> $\text{sub}^{\text{tilisin}}$ <sup>23</sup> | <i>B. amy</i> neutral protease <sup>24</sup><br><i>B. amy</i> $\alpha$ -amylase <sup>22</sup>       |                               |
| <i>Pseudo-monas</i> | $\text{trp}^{27}$ ( <i>E. coli</i> )<br>$\text{lac}$ ( <i>E. coli</i> )<br>$\text{lac}$ ( <i>E. coli</i> )                         | IAA addition,<br>or tryptophan depletion<br>IP1G                               |  |   | phospholipase C <sup>28</sup><br>exotoxin A <sup>28</sup>   | sulfonamide <sup>30</sup><br>streptomycin <sup>30</sup>   | <i>trp</i> ( <i>E. coli</i> ) |
| <i>Yeast</i>        | $\text{gal}^{31}$ , $\text{gal}^{32}$<br>$\text{adh}^{33}$ , $\text{adh}^{34}$<br>$\text{pho}^5$                                   | Glucose depletion<br>and galactose<br>Glucose depletion<br>Phosphate depletion | <i>Cyc1</i><br><i>Una</i><br>$\alpha$ -factor<br><i>Sec 2</i>    |   | Invertase <sup>36</sup><br>Acid phosphatase <sup>36</sup><br>$\alpha$ -factor   | <i>ura</i> <sup>37</sup><br><i>Leu 2</i> <sup>38</sup><br><i>His 3</i><br><i>lap 1</i>              |                               |

\*non-regulated

1 Backman K, Ptashne M and Gilbert W Proc Natl Acad Sci USA 73: 4174-4178 (1976)

2. de Boer H.A., Comstock, L.J. and Vasser, M. *Proc. Natl. Acad. Sci. USA* 80, 21-25 (1983).
- 3 Shimatake H. and Rosenberg, M. *Nature* 292, 128-132 (1981).
- 5 4 Derom, C., Gheysen, D. and Fiers, W. *Gene* 17, 45-54 (1982).
- 5 Halilewell, R.A. and Emtage, S. *Gene* 9, 27-47 (1980).
- 6 Brosius, J., Dull T.J., Sleeter, D.D. and Noller, H.F. *J. Mol. Biol.* 148, 107-127 (1981).
- 10 7 Normanly, J., Ogden, R.C., Horvath, S.J. and Abelson, J. *Nature* 321, 213-219 (1986).
- 8 Belasco, J.G., Nilsson, G., von Gabain, A. and Cohen, S.N. *Cell* 45, 245-251 (1986).
- 15 9. Schmeissner, U., McKenney, K., Rosenberg M. and Court, D. *J. Mol. Biol.* 176, 39-53 (1984).
- 10 Mott, J.E., Galloway, J.L. and Platt, T. *EMBO J.* 4, 1887-1891 (1985).
- 11 Koshland, D. And Botstein, D. *Cell* 20, 749-760 (1980).
- 20 12. Movva, N.R., Kakamura, K. and Inouye, M. *J. Mol. Biol.* 143, 317-328 (1980).
13. Surin, B.P., Jans, D.A., Fimmel, A.L., Shaw, D.C., Cox, G.B. and Rosenberg, H. *J. Bacteriol.* 157, 772-773 (1984).
- 25 14. Sutcliffe, J.G. *Proc. Natl. Acad. Sci. USA* 75, 3737-3741 (1978).
- 15 Peden, K.W.C. *Gene* 22, 277-280 (1983).
- 30 16. Alton, N.K. and Vapnek, D. *Nature* 282, 864-869 (1979).
17. Yang, M., Galizzi, A., and Henner, D. *Nuc. Acids Res.* 11(2), 237-248 (1983).
18. Wong, S.-L., Price C.W., Goldfarb, D.S., and Doi, R.H. *Proc. Natl. Acad. Sci. USA* 81, 1184-1188 (1984).
- 35 19. Wang, P.-Z. and Doi, R.H. *J. Biol. Chem.* 251, 8619-8625. (1984).
20. Lin, C.-K., Quinn, L.A., Rodriguez, R.L. *J. Cell Biochem. Suppl.* (9B), p. 198 (1985).
- 40 21. Vasantha, N., Thompson, L.D., Rhodes, C., Banner, C., Nagle, J., and Filpula, D. *J. Bact.* 159(3), 811-819 (1984).
22. Plava, I., Sarvas, M., Lehtovaara, P., Sibazkov, M., and Kaariainen, L. *Proc. Natl. Acad. Sci. USA* 79, 5582-5586 (1982).
- 45 23. Wong, S.-L., Price, C.W., Goldfarb, D.S., and Doi, R.H. *Proc. Natl. Acad. Sci. USA* 81, 1184-1188 (1984).
24. Sullivan, M.A., Yasbin, R.E., Young, F.E. *Gene* 29, 21-46 (1984).
- 50 25. Vasantha, N., Thompson, L.D., Rhodes, C., Banner, C., Nagle, J., and Filpula, D.J. *Bact.* 159(3), 811-819 (1984).
26. Yansura, D.G. and Henner, D. *J. PNAS* 81, 439-443 (1984).
27. Gray, G.L., McKeown, K.A., Jones, A.J.S., Seeburg, P.H. and Heyneker, H.L. *Biotechnology*, 161-165 (1984).
- 55 28. Lory, S., and Tai, P.C. *Gene* 22, 95-101 (1983).
29. Liu, P.V. *J. Infect. Dis.* 130 (suppl), 594-599 (1974).

- 30 Wood, D.G., Hollinger, M.F. and Tindol, M.B. J. Bact. 145, 1442-1451 (1981).
- 31 St. John, T.P. and Davis, R.W. J. Mol. Biol. 152, 225-315 (1981).
- 32 Hopper, J.E. and Rowe, L.B. J. Biol. Chem. 253, 7566-7569 (1978).
- 33 Denis, C.L., Ferguson, J. and Young, E.T. J. Biol. Chem. 258, 1165-1171 (1983).
- 34 Lutsdorf, L. and Megnet, R. Archs. Biochem. Biophys. 126, 933-944 (1968).
- 35 Meyhack, B., Bajwa, N., Rudolph, H. and Hinnen, A. EMBO J. 6, 675-680 (1982).
- 36 Watson, M.E. Nucleic Acid Research 12, 5145-5164 (1984).
- 37 Gerband, C. and Guerinneau, M. Curr. Genet. 1, 219-228 (1980).
- 38 Hinnen, A., Hicks, J.B. and Fink, G.R. Proc. Natl. Acad. Sci. USA 75, 1929-1933 (1978).
- 39 Jabbar, M.A., Sivasubramanian, N. and Nayak, D.P. Proc. Natl. Acad. Sci. USA 82, 2019-2023 (1985).

#### (i) Pseudomonas Vectors

Several vector plasmids which autonomously replicate in a broad range of Gram negative bacteria are preferred for use as cloning vehicles in hosts of the genus Pseudomonas. Certain of these are described by Tait, R.C., Close, T.J., Lundquist, R.C., Hagiya, M., Rodriguez, R.L., and Kado, C.I. in Biotechnology, May, 1983, pp. 269-275; Panopoulos, N.J. in Genetic Engineering in the Plant Sciences, Praeger Publishers, New York, New York, pp. 163-185 (1981); and Sakaguchi, K. in Current Topics in Microbiology and Immunology 96, 31-45 (1982), each of which is specifically incorporated herein by reference.

One particularly preferred construction would employ the plasmid RSF1010 and derivatives thereof as described by Bagdasarian, M., Bagdasarian, M.M., Coleman, S., and Timmis, K.N. in Plasmids of Medical, Environmental and Commercial Importance, Timmis, K.N. and Puhler, A. eds., Elsevier/North Holland Biomedical Press (1979), specifically incorporated herein by reference. The advantages of RSF1010 are that it is a relatively small, high copy number plasmid which is readily transformed into and stably maintained in both E. coli and Pseudomonas species. In this system, it would be preferred to use the Tac expression system as described for Escherichia, since it appears that the E. coli trp promoter is readily recognized by Pseudomonas RNA polymerase as set forth by Sakaguchi, K. in Current Topics in Microbiology and Immunology 96, 31-45 (1982) and Gray, G.L., McKeown, K.A., Jones, A.J.S., Seeburg, P.H., and Heyneker, H.L. in Biotechnology, Feb. 1984, pp. 161-165, both of which are specifically incorporated herein by reference. Transcriptional activity may be further maximized by requiring the exchange of the promoter with, e.g., an E. coli or P. aeruginosa trp promoter. Additionally, the lacI gene of E. coli would also be included in the plasmid to effect regulation.

Translation may be coupled to translation initiation for any of the Pseudomonas proteins, as well as to initiation sites for any of the highly expressed proteins of the type chosen to cause intracellular expression of the inhibitor.

In those cases where restriction minus strains of a host Pseudomonas species are not available, transformation efficiency with plasmid constructs isolated from E. coli are poor. Therefore, passage of the Pseudomonas cloning vector through an  $r^{-}$   $\text{trp}^{+}$  strain of another species prior to transformation of the desired host, as set forth in Bagdasarian, M. et al. Plasmids of Medical, Environmental and Commercial Importance, pp. 411-422, Timmis and Puhler eds., Elsevier/North Holland Biomedical Press (1979), specifically incorporated herein by reference, is desired.

#### (ii) Bacillus Vectors

Furthermore, a preferred expression system in hosts of the genus Bacillus involves using plasmid pUB110 as the cloning vehicle. As in other host vector systems, it is possible in Bacillus to express the TNF inhibitor of the present invention as either an intracellular or a secreted protein. The present embodiments include both systems. Shuttle vectors that replicate in both Bacillus and E. coli are available for constructing and testing various genes as described by Dubnau, D., Gryczan, T., Contente, S., and Shivakumar, A.G. in Genetic Engineering, Vol. 2, Setlow and Hollander eds., Plenum Press, New York, New York, pp. 115-131 (1980), specifically incorporated herein by reference. For the expression and secretion of the TNF inhibitor from B. subtilis, the signal sequence of alpha-amylase is preferably coupled to the coding region for the protein. For synthesis of intracellular inhibitor, the portable DNA sequence will be



translationally coupled to the ribosome binding site of the alpha-amylase leader sequence

Transcription of either of these constructs is preferably directed by the alpha-amylase promoter or a derivative thereof. This derivative contains the RNA polymerase recognition sequence of the native alpha-amylase promoter but incorporates the lac operator region as well. Similar hybrid promoters constructed from the penicillinase gene promoter and the lac operator have been shown to function in *Bacillus* hosts in a regulatable fashion as set forth by Yansura, D. G. and Henner in *Genetics and Biotechnology of Bacilli*, Ganesan, A.T. and Hoch, J.A., eds. Academic Press, pp. 249-263 (1984), specifically incorporated by reference. The lacI gene of *E. coli* would also be included in the plasmid to effect regulation.

#### (iii) Clostridium Vectors

One preferred construction for expression in *Clostridium* is in plasmid pJU12, described by Squires, C.H. et al., in *J. Bacteriol.* 159:465-471 (1984) and specifically incorporated herein by reference, transformed into *C. perfringens* by the method of Heefner, D.L. et al., as described in *J. Bacteriol.* 159:460-464 (1984), specifically incorporated herein by reference. Transcription is directed by the promoter of the tetracycline resistance gene. Translation is coupled to the Shine-Dalgarno sequences of this same tet<sup>r</sup> gene in a manner strictly analogous to the procedures outlined above for vectors suitable for use in other hosts.

#### (iv) Yeast Vectors

Maintenance of foreign DNA introduced into yeast can be effected in several ways as described by Botstein, D. and Davis, R.W., in *The Molecular Biology of the Yeast Saccharomyces*, Cold Spring Harbor Laboratory, Strathern, Jones and Broach, eds., pp. 607-636 (1982), specifically incorporated herein by reference. One preferred expression system for use with host organisms of the genus *Saccharomyces* harbors the TNF inhibitor gene on the 2 micron plasmid. The advantages of the 2 micron circle include relatively high copy number and stability when introduced into *cir*<sup>-</sup> strains. These vectors preferably incorporate the replication origin and at least one antibiotic resistance marker from pBR322 to allow replication and selection in *E. coli*. In addition, the plasmid will preferably have the two micron sequence and the yeast LEU2 gene to serve the same purposes in LEU2 defective mutants of yeast.

If it is contemplated that the recombinant TNF inhibitors will ultimately be expressed in yeast, it is preferred that the cloning vector first be transferred into *Escherichia coli*, where the vector would be allowed to replicate and from which the vector would be obtained and purified after amplification. The vector would then be transferred into the yeast for ultimate expression of the TNF inhibitor.

#### (c) Mammalian Cells

The cDNA for the TNF inhibitor will serve as the gene for expression of the inhibitor in mammalian cells. It should have a sequence that will be efficient at binding ribosomes such as that described by Kozak, in *Nucleic Acids Research* 15:8125-8132 (1987), specifically incorporated herein by reference, and should have coding capacity for a leader sequence (see section 3(a)(vi)) to direct the mature protein out of the cell in a processed form. The DNA restriction fragment carrying the complete cDNA sequence can be inserted into an expression vector which has a transcriptional promoter and a transcriptional enhancer as described by Guarente, L. in *Cell* 52:303-305 (1988) and Kadonaga, J.T. et al., in *Cell* 51:1079-1090 (1987), both of which are specifically incorporated herein by reference. The promoter may be regulatable as in the plasmid pMSG (Pharmacia Cat. No. 27450601) if constitutive expression of the inhibitor is harmful to cell growth. The vector should have a complete polyadenylation signal as described by Ausubel, F.M. et al. in *Current Protocols in Molecular Biology*, Wiley (1987), specifically incorporated herein by reference, so that the mRNA transcribed from this vector is processed properly. Finally, the vector will have the replication origin and at least one antibiotic resistance marker from pBR322 to allow replication and selection in *E. coli*.

In order to select a stable cell line that produces the TNF inhibitor, the expression vector can carry the gene for a selectable marker such as a drug resistance marker or carry a complementary gene for a deficient cell line, such as a dihydrofolate reductase (dhfr) gene for transforming a dhfr cell line as described by Ausubel et al., *supra*. Alternatively, a separate plasmid carrying the selectable marker can be cotransformed along with the expression vector.

### 7. Host Cells/Transformation

The vector thus obtained is transferred into an appropriate host cell. These host cells may be microorganisms or mammalian cells.

## (c) Microorganisms

It is believed that any microorganism having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen. After a host organism has been chosen, the vector is transferred into the host organism using methods generally known to those of ordinary skill in the art. Examples of such methods may be found in *Advanced Bacterial Genetics* by R W Davis *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, New York, (1950) which is specifically incorporated herein by reference. It is preferred, in one embodiment, that the transformation occur at low temperatures as temperature regulation is contemplated as a means of regulating gene expression through the use of operational elements as set forth above. In another embodiment, if osmolar regulators have been inserted into the vector, regulation of the salt concentrations during the transformation would be required to insure appropriate control of the foreign genes.

It is preferred that the host microorganism be a facultative anaerobe or an aerobe. Particular hosts which may be preferable for use in this method include yeasts and bacteria. Specific yeasts include those of the genus Saccharomyces, and especially Saccharomyces cerevisiae. Specific bacteria include those of the genera Bacillus, Escherichia, and Pseudomonas, especially Bacillus subtilis and Escherichia coli. Additional host cells are listed in Table I *supra*.

## (d) Mammalian Cells

The vector can be introduced into mammalian cells in culture by several techniques such as calcium phosphate-DNA coprecipitation, electroporation, or protoplast fusion. The preferred method is coprecipitation with calcium phosphate as described by Ausubel *et al.*, *supra*.

Many stable cell types exist that are transformable and capable of transcribing and translating the cDNA sequence, processing the precursor TNF inhibitor and secreting the mature protein. However, cell types may be variable with regard to glycosylation of secreted proteins and post-translational modification of amino acid residues, if any. Thus, the ideal cell types are those that produce a recombinant TNF inhibitor identical to the natural molecule.

8. Culturing Engineered Cells

The host cells are cultured under conditions appropriate for the expression of the TNF inhibitor. These conditions are generally specific for the host cell, and are readily determined by one of ordinary skill in the art in light of the published literature regarding the growth conditions for such cells and the teachings contained herein. For example, *Bergey's Manual of Determinative Bacteriology*, 5th Ed., Williams & Wilkins Company, Baltimore, Maryland, which is specifically incorporated herein by reference, contains information on conditions for culturing bacteria. Similar information on culturing yeast and mammalian cells may be obtained from Pollack, R. *Mammalian Cell Culture*, Cold Spring Harbor Laboratories (1975) specifically incorporated herein by reference.

Any conditions necessary for the regulation of the expression of the DNA sequence, dependent upon any operational elements inserted into or present in the vector, would be in effect at the transformation and culturing stages. In one embodiment, cells are grown to a high density in the presence of appropriate regulatory conditions which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental conditions are altered to those appropriate for expression of the DNA sequence. It is thus contemplated that the production of the TNF inhibitor will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant TNF inhibitor will be harvested at some time after the regulatory conditions necessary for its expression were induced.

9. Purification

## (a) TNF inhibitor Produced From Microorganisms.

In a preferred embodiment of the present invention, the recombinant TNF inhibitor is purified subsequent to harvesting and prior to assumption of its active structure. This embodiment is preferred as the inventors believe that recovery of a high yield of re-folded protein is facilitated if the protein is first purified. However, in one preferred, alternate embodiment, the TNF inhibitor may be allowed to refold to assume its active structure prior to purification. In yet another preferred, alternate embodiment, the TNF inhibitor is present in its re-folded, active state upon recovery from the culturing medium.

In certain circumstances, the TNF inhibitor will assume its proper, active structure upon expression in the host microorganism and transport of the protein through the cell wall or membrane or into the periplasmic space. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. If the TNF inhibitor does not assume its proper active structure, any disulfide bonds which have formed

and/or any noncovalent interactions which have occurred will first be disrupted by denaturing and reducing agents: for example, guanidinium chloride and beta-mercaptoethanol, before the TNF inhibitor is allowed to assume its active structure following dilution and oxidation of these agents under controlled conditions.

For purifications prior to and after refolding, some combinations of the following steps is preferably used, anion exchange chromatography (monoQ or DEAE-Sephacrose), gel filtration chromatography (superose), chromatofocusing (MonoP) and hydrophobic interaction chromatography (octyl or phenyl sepharose). Of particular value will be affinity chromatography using TNF (described in Example 1).

#### (b) TNF inhibitor Produced from Mammalian Cells.

TNF inhibitor produced from mammalian cells will be purified from conditioned medium by steps that will include ion exchange chromatography and affinity chromatography using TNF as described in Example 1. It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

As indicated previously, the TNF inhibitors of the present invention are contemplated for use as therapeutic agents and thus are to be formulated in pharmaceutically acceptable carriers. In one embodiment of the present invention, the TNF inhibitors may be chemically modified to improve the pharmacokinetic properties of the molecules. An example would be the attachment of the TNF inhibitors to a high molecular weight polymeric material such as polyethylene glycol. In addition, interleukin-1 inhibitors may be administered in conjunction with the TNF inhibitors. This combination therapeutic will be especially useful in treatment of inflammatory and degenerative diseases.

The following examples illustrate various presently preferred embodiments of the invention claimed herein. All papers and references cited in the Examples that follow are specifically incorporated herein by reference.

### Example 1. Protein Preparation

#### A. Materials

The gene for TNF alpha (TNFa) was purchased from British Biotechnology, Limited, Oxford, England. DEAE-Sephacrose CL-6B resin and Mono-Q HR5/5, HR10/10 FPLC columns were purchased from Pharmacia, Inc., Piscataway, New Jersey. Affigel-15 resin, and BioRad protein assay kit were purchased from BioRad, Richmond, California. Tween 20, ammonium bicarbonate, sodium phosphate, PMSF, sodium bicarbonate, dithiothreitol crystal violet and actinomycin D were purchased from Sigma Chemical Company, St. Louis, Missouri. Endoproteinase Lys-C, Endoproteinase Asp-N and TRIS were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Hexafluoroacetone was purchased from ICN Biomedicals, Costa Mesa, California. Cyanogen bromide, trifluoroacetic acid, and guanidine hydrochloride were purchased from Pierce Chemicals, Rockford, Illinois. Acetonitrile and HPLC water were purchased from J.T. Baker Chemical Company, Phillipsburg, New Jersey. Urea was purchased from Bethesda Research Laboratories, Gaithersburg, Maryland. [<sup>3</sup>H]-iodoacetic acid was purchased from New England Nuclear, Boston, Massachusetts. [<sup>125</sup>I]-TNFa was purchased from Amersham, Arlington Heights, Illinois. Recombinant human TNFa was purchased from Amgen, Thousand Oaks, California. C8-reverse phase columns (25 cm x 4.6 mm) were obtained from Synchrom, Inc., Lafayette, Indiana. A C8-microbore reverse phase column (7 micron, 22 cm x 2.1 mm) was obtained from Applied Biosystems, Foster City, California. Coming 96-well microtiter plates were purchased from VWR Scientific, Batavia, Illinois. McCoy's 5A media and fetal bovine serum were purchased from Gibco, Grand Island, New York. RPMI-1 1640 media and L-glutamine were purchased from Mediatech, Herndon, Virginia. Trypsin was purchased from K. C. Biologicals, St. Lenexa, Kansas. ME180, U937 and L929 cell lines were obtained from American Type Culture Collection, Rockville, Maryland.

#### B. Assays for the TNF inhibitor

Two types of assays were used to identify the TNF inhibitor. One of them is a cytotoxicity assay. The other is a gel shift assay.

##### 1. Cytotoxicity Assay

The cytotoxicity assay was performed with actinomycin D-treated ME180 cells and L929 cells as described by Ostrove and Gifford (Proc. Soc. Exp. Biol. Med. 160 354-358 (1979)) and Aggarwal and Essalu (J. Biol. Chem. 252 10000-10007 (1987)). L929 cells (CCCL, American Type Culture Collection) cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum. Confluent cultures were treated briefly with 0.25% trypsin in physiological

solution containing 5mM EDTA and resuspended in a fresh medium. Approximately  $2 \times 10^4$  trypsinized cells per well were plated in 96-Well plates (Corning) and incubated for 24 hours at 37°C. Then actinomycin D was added to a final concentration of 0.25 µg per ml. After two hours, samples containing TNF and TNF inhibitor were added to the wells and incubation was continued overnight at the same temperature. After microscopic evaluation, the medium was decanted, and the wells were rinsed with PBS. The wells were then filled with a solution of 0.1% crystal violet, 10% formaldehyde and 10mM potassium phosphate, pH 6.0 for 5 min, washed thoroughly with water and dried. The dye was extracted with 0.1M sodium citrate in 50% ethanol, pH 4.2. The absorbance of the dye in viable cells was determined at 570 nm using a Kinetic microplate reader (Molecular Devices Corp., CA). An example of this assay is shown in Figure 1. In the presence of TNF inhibitor, the cytotoxic effect of TNF was reduced.

## 2. Gel Shift Assay

The gel shift assay involves the use of a native polyacrylamide gel electrophoresis system. This native 4% gel electrophoresis was performed according to Hedrick and Smith (Arch. Biochem. and Biophysics 126: 155-164 (1968)). The iodinated TNF (Amersham) was mixed with the TNF inhibitor from Example 1.C. after C8 chromatography and incubated for 30 min. to 2 hours. This mixture, along with the iodinated TNF alone, were loaded onto the 4% native gel and electrophoresed. After the gel was fixed with 10% acetic acid and washed, a film was placed for radioautography. As shown in Figure 2, the complex of TNF and TNF inhibitor migrates differently from the TNF by itself. This gel shift assay was used to determine which fractions contain TNF inhibitor in the eluates of DEAE CL6B column chromatography.

## C. Purification of the 30kDa TNF Inhibitor

Twenty liters of urine from a patient diagnosed with renal dysfunction was concentrated to 200 ml with an Amicon YM5 membrane. The concentrate was then dialyzed at 4°C against 0.025 M Tris-Cl, pH 7.5, and subsequently centrifuged in a JA14 rotor at 10,000 rpm for 30 minutes. The supernatant was then loaded onto a 40 x 4.5 cm DEAE Sepharose CL-6B column equilibrated with 0.025 M Tris-Cl, pH 7.5 and extensively rinsed with equilibration buffer until the OD<sub>280</sub> of the effluent returned to baseline. Chromatography was accomplished using a linear gradient from 0-0.05 M sodium chloride in 0.025 M Tris-Cl pH 7.5 and monitored by OD<sub>280</sub>. Column fractions were collected, and assayed for TNF inhibitor activity using the native gel assay. The TNF inhibitor eluted in a rather sharp peak at 80mM NaCl.

Figure 6A shows the OD<sub>280</sub> profile of the DEAE Sepharose CL-6B chromatography of 20 l urine. Figure 6B shows the autoradiograph of the corresponding native gel assay indicating a peak of the TNF inhibitor at fractions 57-63, which is about 80mM NaCl.

The TNF inhibitor was further purified using a TNF affinity column. Recombinant TNF was expressed in BL21/DE3 at about 10-20% total cell protein. The cell pellet was French-pressed at 20,000 psi and the soluble material dialyzed at 4°C against 0.025 M Tris-Cl pH 8.0. The dialyzed lysate was 0.2 micron-filtered and loaded onto a Mono-Q FPLC column equilibrated with 0.025 M Tris-Cl pH 8.0. A linear gradient from 0 to 0.5 M NaCl in 0.025 M Tris-Cl pH 8.0 was run and monitored by OD<sub>280</sub>. One ml fractions were collected and analyzed for purity by SDS-PAGE. The subsequent TNFa pool was about 95% pure based on Coomassie-stained SDS-PAGE and was fully active based on a Bradford protein assay, using lysozyme as a standard, and an ME180 bioassay, using Amgen's TNFa as a standard (Bradford, M. Annal. Biochem. 72: 248-254 (1976)).

TNFa was concentrated in an Amicon Centriprep-10 to about 25 mg/ml, dialyzed against 100 mM NaHCO<sub>3</sub>, pH 8.5, and coupled to Affigel-15 resin at 25 mg TNF/ml resin. A coupling efficiency of greater than 80% yielded a high capacity resin which was used to further purify the TNF inhibitor.

PMSF, at a final concentration of 1-4 mM, was added to the DEAE CL-6B pool and applied to a 4 x 1 cm TNF affinity column equilibrated at 4°C with 0.025 M Tris-Cl pH 7.5 at a flow rate of 0.1 ml/min. The column was then rinsed with 0.025 M Tris-Cl pH 7.5 until the OD<sub>280</sub> of the effluent returned to baseline. The column was subsequently eluted with 0.05 M NaPhos, pH 2.5 and monitored by OD<sub>280</sub>. Figure 7 shows the OD<sub>280</sub> profile of the 0.05 M NaPhos pH 2.5 elution from the TNF affinity column.

The TNF inhibitor was purified to homogeneity by reverse phase HPLC on a Syncropak RP-8 (C8) column. The OD<sub>280</sub> peak from the TNF affinity column was pooled and immediately loaded onto a RP-8 column, equilibrated with 0.1% TFA/H<sub>2</sub>O. A linear 1%/min gradient of 0.1% TFA/acetonitrile was run from 0-50%, and monitored by OD<sub>215</sub> and OD<sub>280</sub>. Fractions were collected and assayed for bioactivity using L929 cells and the native gel assay described in Example 1.B. Both of these assays indicate bioactivity at fractions 23-32 which corresponds to a peak of OD<sub>215</sub> and OD<sub>280</sub> eluting at 12% acetonitrile.

Figures 8A and 8B show the chromatographic profile of the TNF affinity pool on a Syncropak RP-8 column with the corresponding bioactivity from the L929 cytotoxicity assay. Figure 8B shows a silver stained 15% reducing SDS-PAGE of the RP-8 pool indicating a single band at 30kDa.

D. Characterization of the Protein Component of 30kDa TNF Inhibitor

30kDa TNF inhibitor is a glycoprotein as was detected using Concanavalin A-Peroxidase after the protein was transferred onto the nitrocellulose filter. This method is a modification of Wood and Sarinana (Analytical Biochem 59 320-322 (1975)) who identified glycoproteins on an acrylamide gel directly. The peroxidase staining of glycoprotein was performed by using peroxidase conjugated Con A or non-conjugated Con A. When non-conjugated Con A was used, the nitrocellulose filter was incubated for one hour in a solution containing Con A (0.5 mg/ml, Miles Laboratory) in phosphate buffer, pH 7.2 (PBS), then washed 3 x 5 min. in PBS. The washed filter was incubated in horseradish peroxidase (0.1 mg/ml, Sigma Chemical) for one hour. After 3 x 15 min. in PBS the filter was immersed in a solution containing 3 mg/ml 4-chloro-1-naphthol (Sigma Chemical) and 12.5  $\mu$ l/ml of hydrogen peroxide until the color was developed. Glycoprotein was seen as a purple color. A photograph was made as soon as the filter was developed as shown in Figure 3.

Chemical deglycosylation of TNF inhibitor was carried out by the method of Edge, Faltynek, Hof, Reichert and Weber (Analytical Biochem. 116, 131-137 (1981)). A mixture of 0.25 ml anisole (Eastman Kodak) and 0.5 ml of (trifluoromethanesulfonic acid (Eastman Kodak) was cooled to 4°C, then 1-200 ng of dry TNF inhibitor were dissolved in 3  $\mu$ l of this mixture. The tube was flashed with nitrogen, then incubated for 30 min. at room temperature. This deglycosylated protein was analyzed on SDS-PAGE (Figure 4). The molecular weight of chemical treated TNF inhibitor is about 18,000 dalton. A band at 14,000 was seen also, but this may be a proteolytic fragment of deglycosylated TNF inhibitor.

The enzymatic deglycosylation using N-glycanase was performed following the manufacturer's protocol (Genzyme Corp.) except TNF inhibitor was incubated with N-glycanase for 5 to 6 hours instead of overnight. The molecular weight of the deglycosylated form of denatured TNF inhibitor is shown to be about 20,000 dalton (Figure 5). When the inhibitor is not denatured prior to deglycosylation, the molecular weight of the deglycosylated protein is about 26,000 dalton.

E. Deglycosylated 30kDa TNF-inhibitor binds to TNF

Radiolabeled TNF inhibitor (30kDa) was treated with TFMSA (trifluoromethanesulfonic acid) in order to remove carbohydrates, and the TFMSA was separated from the protein by HPLC. The protein fraction was mixed with TNF-affigel for one hour at 4°C, and all unbound material was removed by centrifugation. The TNF-affigel was washed extensively with 50mM NaPO<sub>4</sub>, pH 2.5. Radioactivity in each fraction was counted and also analyzed on a SDS-PAGE. Non-specific binding of TNF inhibitor was measured using anhydrochymotrypsin affigel. The results are shown in Table 2. These results indicate that deglycosylated TNF inhibitor (30kDa) binds to TNF.

TABLE 2

| Sample                | Type of Affinity | Count (CPM)   |               |
|-----------------------|------------------|---------------|---------------|
|                       |                  | Flow Through  | Eluate        |
| Native TNF-INH        | TNF              | 49401 (55.0%) | 40014 (45.0%) |
| Native TNF-INH        | Anhy CT          | 80000 (98.0%) | 1799 (2.0%)   |
| TFMSA-Treated TNF-INH | TNF              | 13369 (73.0%) | 4908 (27.0%)  |
| TFMSA-Treated TNF-INH | Anhy CT          | 15682 (94.0%) | 926 (6.0%)    |

In another experiment, radiolabeled TNF inhibitor (30kDa) was reduced, then deglycosylated with N-glycanase. After deglycosylation, the material was incubated with 13 mM oxidized glutathione (GSSG) for 10 minutes at room temperature, and diluted 5 fold with 50mM Tris. Cysteine was then added to a final concentration of 5mM. The material was incubated at 4°C for 16 hours then mixed with a TNF-affigel for one hour at 4°C. Unbound material was removed, and the gel was washed extensively with 50mM Tris-HCl, pH 7.5. The bound material was eluted with 50mM NaPO<sub>4</sub>, pH 2.5. Radioactivity in each fraction was analyzed, and a SDS-PAGE was performed for each fraction. As seen in Table 3 and Figure 18, the deglycosylated and reoxidized TNF inhibitor also binds to TNF.

TABLE 3

| Sample                              | Type of Affinity | Count (CPM)   |               |
|-------------------------------------|------------------|---------------|---------------|
|                                     |                  | Flow Through  | Eluate        |
| Native TNF-INH                      | TNF              | 18281 (60.0%) | 12603 (40.0%) |
| Native TNF-INH (reduced/reoxidized) | TNF              | 28589 (94.0%) | 1964 (6.0%)   |
| TFMSA-Treated (reduced/reoxidized)  | Anhy CT          | 31371 (93.70) | 421 (1.3%)    |

TABLE 3 (continued)

| Sample                                      | Type of Affinity | Count (CPM)   |              |
|---|------------------|---------------|--------------|
|   |                  | Flow Through  | Eluate       |
| Deglycosylated TNF-INH (reduced/reoxidized) | TNF              | 25066 (85.0%) | 4305 (15.0%) |
| Deglycosylated TNF-INH (reduced/reoxidized) | Anhy CT          | 29619 (98.4%) | 495 (1.6%)   |

Example 2 Sequencing of 30kDa TNF Inhibitor

N-terminal sequences were determined using Applied Biosystems Protein Sequencers, models 470 and 477. Prior to sequencing, peptides generated from a variety of proteolytic enzymes were purified on an Applied Biosystems C8-microbore HPLC column (22 cm x 2.1 mm).

A. Amino Terminal Sequencing

Approximately 250 pmoles of reverse phase (RP-8) purified TNF inhibitor were applied directly to a polybrene filter and subjected to automated Edman degradation. The resulting sequence information yielded the first 30 amino acids of the molecule.

B. Endoproteinase Lys-C Digestion of Native Protein

Approximately 250 pmoles (5 ug) of reverse-phase purified TNF inhibitor was digested with 1 ug of endoproteinase Lys-C. The 12 hour digestion at 25°C was carried out in the presence of 1M urea, 0.01% Tween 20, and 150 mM  $\text{NH}_2\text{HCO}_3$ , pH 8.0. Prior to peptide purification the digest was reduced by incubation for 1 hour following addition of 50-fold molar excess of dithiothreitol, or reduced and alkylated by a further one hour incubation at 37°C using a two-fold molar excess of [ $^3\text{H}$ ]-iodoacetic acid over dithiothreitol. Figure 9A shows the reverse phase HPLC pattern of this digestion. Figure 9B shows the reverse phase HPLC pattern of this digest followed by alkylation.

C. Endoproteinase Asp-N Digestion of Native Protein

Approximately 250 pmol (5ug) of reverse phase purified TNF inhibitor was digested with 0.5-2.5 ug endoproteinase Asp-N. The 12-18 hour digest at 37°C was carried out in the presence of 1M guanidine-HCl, 0.01% Tween 20 and 150 mM NaPhos, pH 8.0.

Prior to peptide purification the digest was reduced and alkylated as in Example 2.B. Figure 10 shows the reverse phase HPLC pattern of two such digests.

D. Reduction Carboxymethylation of Protein

The reverse-phase HPLC purified TNF inhibitor was reduced and carboxymethylated with [ $^3\text{H}$ ] iodoacetic acid as described by Glazer, et al., in Chemical Modifications of Proteins, pp. 103-104 (1975) except two successive rounds of reduction followed by alkylation were used. The protein was re-purified by reverse-phase HPLC prior to proteolytic digestion.

E. Endoproteinase V8 Digestion of Reduction Carboxymethylation of Protein

An analytical digest was performed by dissolving 55 pmoles (about 1 ug) of reduced carboxymethylated TNF inhibitor in 150 mM  $\text{NaHCO}_3$ , pH 8.0, and digesting it with 0.2 ug V8 protease for 18 hours at 25°C. Reverse-phase HPLC (Figure 11A) revealed three sequenceable peptides and indicated a larger scale digest was in order. Approximately 220 pmoles (4.5 ug) of reduced carboxymethylated TNF inhibitor was digested with 1 ug V8 protease for 5 hours at 25°C, when an additional 0.5 ug V8 protease was added and the digestions continued for 16 hours. Figure 11B shows the reverse-phase HPLC of the large scale V8 digest.

F. Complete Primary Structure of 30kDa TNF Inhibitor Based on Peptides Sequences and cDNA Sequence

Various peptide fragments were aligned according to the cDNA sequence obtained in Example 4. This is shown in Figure 19. Residues which are not identified by protein sequencing are residue numbers 14, 42, 43, 44, 96, 97, 105, 107, 108, and 110 through 119. The sequence of Gin-Ile-Glu-Asn is apparently the carboxyl terminus of the 30 kDa

## TNF inhibitor

Example 3 30kDa TNF inhibitor is produced by U937 cells stimulated with PMA and PHA

5 The monocyte-like cell line U937 was grown at 37°C in RPMI medium containing 10% fetal calf serum to a cell density of  $1 \times 10^6$  cells/ml. The cells were then removed by centrifugation and resuspended on 5 different 100 cm<sup>2</sup> petri plates at  $2 \times 10^6$  cells/ml in RPMI without serum containing 10 ng/ml of PMA (phorbol 12-myristate 13-acetate) and 5 µg/ml PHA-P (phytohemagglutinin-P). The conditioned medium from one plate was harvested after only 10 minutes of incubation and used as a zero time control. The medium from the remaining plates was successively removed at 24 hours, 48 hours, 72 hours and 96 hours after plating. The protein contained in these samples was concentrated into approximately 400 µl each by Centrprep-10 (Amicon Corp.) treatment. Each 400 µl sample was then mixed with an equal volume of an Affigel-15 (Biorad Corp.) preparation containing approximately 10 mg/ml of purified human recombinant TNFα that had been prepared in our laboratory. This TNFα, prior to being bound to the Affigel-15 resin, had been shown to be bioactive by its toxicity to murein L929 cells.

15 The conditioned medium was incubated at room temperature batchwise with the TNFα affinity resin for 2 hours. The unbound fraction was removed after centrifugation of the resin and the resin was subsequently washed with 1 ml (500 µl, 2x) of PBS (phosphate buffered saline, pH 7.5) containing 0.1% gelatin. Bound material was eluted with a 25 mM solution of monobasic sodium phosphate, pH 2.5 (400 µl, 2x). 40 µl of each of the unbound, washed, and eluted fractions were dried, resuspended in 10 µl of 25 mM Tris pH 7.5, mixed with 2 µl (100 pci) of <sup>125</sup>I-TNFα (400-800 ci/mmole, Amersham) and incubated for 30 minutes at room temperature. These mixtures were then mixed with 5 µl of 40% sucrose and 1 ml of 0.1% bromophenol blue and applied to a 4% native acrylamide gel as described in Example 1 B. The conditioned medium from all samples except the zero control contained TNFα binding activity by this assay as shown in Figure 15.

25 The remaining 300 µl from each sample (1st low pH elution) were applied to a C8 HPLC column and eluted with a linear gradient of acetonitrile over 60 minutes (1%/minute, 1 ml/minute flow rate, 1 ml fractions were collected). Each fraction was dried and resuspended in 50 µl of PBS + 0.1% gelatin. 10 µl of each of these samples was mixed with <sup>125</sup>I-TNFα as above and analyzed by native polyacrylamide gel. TNFα binding activities are detected in fractions corresponding to 33% and 36% acetonitrile as shown in Figure 16.

30 Example 4. Analysis of messenger RNA from PMA/PHA treated U937 cells

U937 cells were grown as described in Example 3 to a density of  $1 \times 10^6$  cells/ml and then resuspended in serum-free medium at  $2 \times 10^6$  cells/ml without or with PMA (10 ng/ml) and PHA (5 µg/ml). Samples were taken at 1 hour +/- PMA/PHA and 17 hours + PMA/PHA only. Total RNA was prepared from the cells by the guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (Analytical Biochemistry 162:156-159, (1987)). Poly A<sup>+</sup> RNA was prepared from total RNA by annealing to oligo dT cellulose (Bethesda Research Labs). Eight micrograms of each poly A<sup>+</sup> RNA was then applied to a 6.6 formaldehyde, 1.2% agarose gel. The RNA within the gel was then blotted to a zeta probe membrane (BioRad). The membrane was treated as described in Example 5 for screening of a human genomic DNA library with oligonucleotide probes.  $1 \times 10^6$  cpm/ml of a labelled single stranded DNA probe (polynucleotide kinase) was added. The sequence of this probe is:

5' TTGTGGCACTTGGTACAGCAAAT 3'

45 and it corresponds to bases 410-433 of the sequence set forth in Figure 13. Following overnight hybridization at 65°C, the membrane was washed once at room temperature in 6 X SSC 0.1% SDS and once at 65°C in the same solution and then exposed to x-ray film for 72 hours. The autoradiogram shown in Figure 17 shows that PMA/PHA treatment of U937 cells in serum-free medium for 1 hour clearly stimulates the expression of the 30kDa TNFα inhibitor messenger RNA and that by 17 hours of treatment this message is virtually absent from the cells. The molecular size of the 30kDa TNFα inhibitor messenger RNA based on this experiment is approximately 2.4 kilobases.

Example 5 Preparation of a human genomic DNA library for 30kDa TNF inhibitor

55 Human genomic DNA was partially digested with Sau3AI and size selected. DNA with an average size of 15 KB was ligated into the BamHI site of bacteriophage lambda Charon 30 (Rimm, D.L., Horness, D., Kucera, J. and Blattner, F.R. Gene 12:301-309 (1980)). Phage were propagated and amplified on E. coli CES 200

A Probes

The four degenerate oligonucleotide hybridization probes listed in Table 4 were synthesized on an Applied Biosystems DNA synthesizer. Each probe mixture consisted of all possible DNA sequences coding for the given peptide sequence.

TABLE 4

| Peptide Name | Peptide Sequence | Probe Name | Probe Sequence               |
|--------------|------------------|------------|------------------------------|
| LysC 18      | KEMGQVE          | TNFBP-P20  | 5' TCNACTCTGNCCATTCTCTCTT 3' |
| LysC 11      | QGKYIHP          | TNFBP-P2'  | 5' CAAGGGNAAAGTATCACATCC 3'  |
| LysC 11      | YNDCPG           | TNFBP-P3'  | 5' TATCAATCGATCTGTCCCNGG 3'  |
| LysC 11      | YIHPQNN          | TNFBP-P4   | 5' TTAGTTTCTGNGGAGTCAGT 3'   |

N = G, A, T, or C.

Oligonucleotides were labeled with [ $\gamma$ - $^{32}$ P] ATP (Amersham Inc., Arlington Heights, IL) and T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) to a specific activity of  $6-9 \times 10^6$  c.p.m./picomole according to manufacturer's instructions.

B. Methodology:

$8.4 \times 10^5$  lambda phage containing human genomic DNA were plated and transferred to duplicate nitrocellulose filters. These filters were hybridized with 1 pMol/ml of probe TNFBP-P2' for 16 hours in a solution containing 1.0 M NaCl, 0.1 M sodium citrate, 2x Denhardt's solution (Denhardt, D.T. Biochem. Biophys. Res. Commun. 23:641-646 (1966)), 0.1% SDS, 0.05% sodium pyrophosphate and 150 ug/ml yeast tRNA at a temperature of 52°C. This temperature is 2°C below the calculated  $T_m$  for the most AT-rich member of the oligonucleotide pool. (Suggs, S.V. in Developmental Biology Using Purified Genes, (Brown, D.D., and Fox, C.F., eds.) Academic Press, New York, pp. 683-693 (1981)). After hybridization, the filters were washed for 45 minutes at ambient temperature with three changes of 1 M NaCl, 0.1 M sodium citrate and 0.5% SDS. A stringent wash of eight minutes was done at the calculated  $T_m$  (i.e., 2°C above hybridization temp) for the most AT-rich member to the pool. Filters were then dried and autoradiographed for 40 hours with one intensifying screen at -70°C.

Eleven positive hybridizing plaques were detected and these were isolated and amplified. The ability of these clones to hybridize to TNFBP-P20, TNFBP-P3' and TNFBP-P4 was tested using similar methodology. One clone (TNFBP-9) hybridized to all four oligonucleotides. This clone was plaque purified and amplified. DNA was prepared from this clone using Lambda-Sorb (Promega Corporation, Madison, WI) and a method described by the manufacturer.

One microgram of this DNA was then digested with Sau3AI and the fragments subcloned into BamHI digested M13 sequencing vector mp 18 (Yanish-Perron, C., Viera, J., and Messing, J. Gene 33:103-119 (1985)). M13 clones were then transferred to duplicate nitrocellulose filters and hybridized to the oligonucleotide probes in Table 4 using conditions previously described. Positive subclones were purified and sequenced (Sanger, F. and Coulson, A.R. J. Mol. Biol. 94:441-448 (1975)) using a modified T4 DNA polymerase (Sequenase, US Biochemical Corp., Cleveland, OH) as described by the manufacturer, and using as primers either the degenerate probes used to identify the clone or sequence obtained using those probes. Among the sequences obtained are those of Subclones TNFBP-M13-Sau3A-P2'-2 and TNFBP-M13-Sau3A-P4 Primers P3', P3', P2', P2 and P4. The sequence data is set forth in Figure 13. The sequence contains DNA coding for at least 48 amino acids of 30kDa TNF inhibitor peptides other than those specified by the probes and therefore confirms that the clone TNFBP9 codes for TNF inhibitor. The sequence also shows that the gene for TNF inhibitor includes at least one intron (GTAGGGGCAA CCCCATTACAG). Finally, this sequence shows that 30kDa TNF inhibitor is synthesized as a precursor protein and that a proteolytic cleavage at the Arg-Asp sequence is required to generate the mature, active protein.



Example 6 Preparation and screening of a cDNA library of mRNA from U937 cells stimulated with PMA/PHA

The experiment described in Example 4 shows that U937 cells treated with PMA/PHA for 1 hour should contain a pool of messenger RNA enriched for the TNF inhibitor (30kDa). Accordingly, a cDNA library was prepared from polyA<sup>+</sup> RNA obtained from U937 cells treated with PMA/PHA as described in Example 4. Double stranded, blunt ended cDNA was obtained from approximately 5 ug of poly A<sup>+</sup> RNA essentially as described by Gubler, U. and Hoffman, B. J. (1983) Gene, 25:263) using lot tested reagents (Amersham, Arlington Heights, IL) according to procedures recommended by the manufacturer. Approximately 1 ug of double stranded cDNA obtained was treated with the enzyme EcoRI methylase and EcoRI linkers having the sequence: d(pCCGGAATTCCGG) (New England Biolabs, Beverly, MA) were attached via T4 DNA ligase followed by digestion with endonuclease EcoRI. This DNA was then ligated into a lambda-bacteriophage cloning vector gt10 (Young, R.A., and Davis, R.W. (1983) Proc. Natl. Acad. Sci. USA, 80:1194-1195) that had been digested with EcoRI and the product packaged into infective lambda-bacteriophage particles using lambda-DNA packaging extracts (Gigapack II Gold) obtained from Stratagene (La Jolla, CA) according to their protocol. This lambda-lysate (cDNA library) was then used to infect E. coli strain C600 hflA and it was shown that the library contained approximately  $2.5 \times 10^6$  recombinant members.

Approximately  $4 \times 10^5$  members of this library were plated on E. coli strain C600 hflA ( $5 \times 10^4$  p.f.u./plate). Duplicate lifts to nitrocellulose were made and the filters were treated as described in Example 5 for screening of the human genomic library. The DNA on the filters was then hybridized to the same <sup>32</sup>P labelled probe as described in Example 4 except that the temperature of incubation was 42°C. From  $4 \times 10^5$  recombinant phage plated, 3 duplicate plaques hybridized to this probe. These were further reisolated and probed as above and with an additional synthetic probe having the sequence:

**5' CCCCCGGGCCTGGACAGTCATTGTA 3'**

This probe corresponds to bases 671-694 of the human genomic TNF inhibitor clone shown in Figure 13. Both probes hybridized to all three plaques identified with the first.

After plaque purification DNA was prepared from these three clones and subcloned into the EcoRI site of M13 vectors MP18 and MP19 as described in Example 5. Each of these cDNAs consist of two EcoRI fragments one of approximately 800 bp common to all three clones and another 1300 bp, 1100 bp or 1000 bp depending on the clone. The likely origin of the unique EcoRI fragments in each clone is incomplete elongation by the enzyme reverse transcriptase during 1st strand synthesis of the cDNA. Therefore, those EcoRI fragments likely represent the 5' end of the TNF inhibitor mRNA and the 800 bp fragment the 3' end. This is confirmed by the DNA sequence obtained for these fragments as described below.

From the EcoRI subclones of the cDNA described above the entire sequence of the 2100 bp cDNA was obtained. The dideoxy nucleotide chain termination method of sequencing was used (Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. 94:441-448). The modified T7 DNA polymerase, Sequenase (U.S. Biochemical, Cleveland, OH) was used as the elongation enzyme as described by the supplier. Sequencing primers were synthetic oligonucleotides prepared from the human genomic sequence of the TNF inhibitor as shown in Figure 13 or sequences obtained using those primers. Figure 20 shows the translated sequence derived from one of the cDNA clones. This sequence corresponds to that obtained by protein sequence data as described in Figure 19. The entire sequence of the human 30kDa TNF inhibitor cDNA from clone lambda-gt10-7ctnfbp is shown in Figure 21.

Example 7. Expression of the 30kDa TNF inhibitor cDNA in Escherichia coli

The portion of the TNF inhibitor (30kDa) cDNA gene coding for the soluble TNFα binding activity has been prepared for expression in E. coli as described below.

Because the protein coding sequence defining the C-terminal portion of the urine derived TNF inhibitor (sequence QIEN, base 771 Figure 20) is not followed by a termination codon in the cDNA sequence, one was added by oligonucleotide directed in vitro mutagenesis (Biorad, Richmond, CA). An M13MP19 clone of the 1300 bp EcoRI fragment from the clone lambda-gt107ctnfbp, was hybridized with the synthetic oligonucleotide:

**5' CTACCCAGATTGAGAATTAAGCTTAAGGGCACTGAGGAC 3'**

After 2nd strand synthesis and transfection into an appropriate host, mutant clones were identified by hybridization to the above described mutagenic oligonucleotide. The molecular identity of the clones so identified was confirmed by DNA sequencing as described (Example 5). Next, a 468 bp fragment defined by Styl (position 303) and HindIII defining

the C-terminus of the protein was removed from the Rf form as a mutagenized clone and inserted into *E. coli* expression plasmid containing the *lacI* promoter (DeBoer H A et al (1993) *Proc. Natl. Acad. Sci. USA* 90 21-25). This construction was accomplished by use of the synthetic double strand adapter sequence.

5' GATCCGATCTTGGAGGATGATTAAATGGACAGCGTTTGCCCC 3'  
GCTAGAACCTCCTACTAATTTACCTGTTCGCAAACGGGGGTTT

This adapter translationally couples the TNF inhibitor gene (truncated form as described above) to the first 12 codons of the bacteriophage T7 gene 10. The DNA sequence of this construct from the point of translation initiation at gene 10 through the adapter sequence is shown in Figure 22. A methionine codon (ATG) is necessarily added to the TNF inhibitor gene sequence for expression in *E. coli*. This plasmid is called pTNFiX-1.

The predicted molecular weight of this protein is approximately 17 600kDa a molecular weight that is very close to the deglycosylated native TNF inhibitor (30kDa).

#### Example 8 Purification of active TNF inhibitor (30kDa) from *Escherichia coli*

Cells from one liter of *E. coli* culture (pTNFiX-1JM1071on-) grown under induced condition for 2 hours were re-suspended in 10 ml of 50mM Tris-HCl, pH 7.5 containing 2mM EDTA (TE buffer) and French pressed at 20,000 psi, at 4°C. The material was centrifuged at 20,000g for 10 min. The resulting pellet was washed once with TE-buffer. The washed pellet was resuspended in 2 ml of 6M Guanidine-HCl and incubated at room temperature for 10 min. After the incubation, 20 ul of 500 mM DTT was added and the mixture was incubated at room temperature for another 30 min. The material which remained insoluble after this treatment was removed by centrifugation at 20,000g for 15 min. 120 ul of 500 mM oxidized glutathione was added to the supernatant, and the mixture was incubated at room temperature for 10 min. This material was then diluted in 20 ml of 0.6% Tri base solution, and 220 ul of 500 mM cysteine was added. The incubation was continued for another 16 hours at 4°C. After 16 hours of incubation, some insoluble residue was observed. This insoluble material was removed by centrifugation at 20,000g for 20 min. The resulting supernatant was dialyzed against 50mM Tris-HCl pH 7.5 for 16 hours at 4°C, then centrifuged at 20,000g for 10 min. PMSF at a final concentration of 4mM was added to this supernatant and this material was loaded onto a TNF-affinity column (7 x 2cm) at a flow rate of 0.1 ml per min. This column was extensively washed with 50mM Tris-HCl pH 7.5, and bound proteins were eluted with 50mM NaPO<sub>4</sub>-HCl pH 2.5. The pH 2.5 eluate was loaded onto an RP8 column which was previously equilibrated with 0.1% TFA/H<sub>2</sub>O. TNF inhibitor was eluted with a linear gradient of 0.1% TFA/acetonitrile at 1%/min. (Figure 25). Fractions were analyzed on SDS-PAGE (Figure 26), and cytotoxicity assay was performed (Figure 25) to localize the TNF inhibitor. The *E. coli*-produced TNF inhibitor (30kDa) migrates to about 20 kDa, since it is not glycosylated. Fractions number 30 through 35 contain TNF inhibitor. The amino terminal sequence of this material shows that the *E. coli* produced TNF inhibitor has the following sequence:

Met-Asp-Ser-Val-( )-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-  
Gln-Asn-Asn-Ser-

By using this procedure, about 40 ug of TNF inhibitor (30kDa) was obtained from one liter of the culture. The yield was about 2 to 3%. The yield can be increased to over 50% by purifying the TNF inhibitor before refolding.

#### Example 9 Expression of genes encoding 30kDa TNF inhibitor in animal cells

Animal-cell expression of TNF inhibitor requires the following steps:

- Construction of an expression vector
- Choice of host cell lines
- Introduction of the expression vector in host cells.
- Manipulation of recombinant host cells to increase expression levels of TNF- $\beta$

1. TNF inhibitor expression vectors designed for use in animal cells can be of several types including strong constitutive expression constructs, inducible gene-constructs as well as those designed for expression in particular cell types. In all cases, promoters and other gene regulatory regions such as enhancers (inducible or not) and polyade-

nylation signals are placed in the appropriate location in relation to the cDNA sequences in plasmid-based vectors. Two examples of such constructs follow.

A construct using a strong constitutive promoter region can be made using the cytomegalovirus (CMV) immediate early gene control signals. This plasmid can be constructed using standard molecular biological techniques (Maniatis et al. Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, 1982) and resulting in the plasmid shown in Figure 23 (pCMVXV beta TNFBPstopA). The SV40 origin of replication is included in this plasmid to facilitate its use in COS cells for transient expression assays. This particular construct contains the CMV immediate early promoter and enhancer as described by Boshart, et al., (Cell 41:521-530, 1985) followed by the rabbit B-globin second intron (see van Ooyen et al., Science 206:337-344, 1979) which is flanked by BamHI and EcoRI restriction sites. This intron is included because expression levels have been shown to be increased when introns are included in the transcribed regions of some expression vectors (Buckman and Berg, Mol. Cell. Biol. 3:4395-4405, 1988). The polyadenylation signal is provided by simian virus 40 (SV40) sequences (map coordinates 2589-2452; see Reddy, et al., Science 200:494-502, 1978). The 30kDa TNF inhibitor cDNA sequences have been modified as follows: the extensive region located 3' of the C-terminus of the purified TNF inhibitor from human urine has been deleted and a stop codon has been engineered into the position just following the C-terminal asparagine. The unmodified 30kDa TNF inhibitor cDNA sequences in an analogous vector have been inserted into COS cells and been shown to increase the TNF binding activity of such cells.

The second construct (see Figure 24) (pSVXVTNFBP stop A) uses the strong constitutive promoter region from the SV40 early gene in an arrangement such as that found in the plasmid pSV2CAT (Gorman, et al., Mol. Cell. Biol. 2:1044-1051, 1982). This plasmid should be manipulated in such a way as to substitute the TNF inhibitor cDNA for the chloramphenicol acetyltransferase coding sequences using standard molecular biological techniques. Once again, the TNF inhibitor cDNA has been modified as described above for the CMV promoter construct. The SV40 early promoter region includes sequences from the HindIII site to the BamHI site (map coordinates 5090-188; see Reddy et al., Science 200:494-502, 1978) and the SV40 polyadenylation signal is as described above for the CMV construct.

2. Two animal cell lines have been used to express TNF inhibitor using the vectors described above to produce active protein. Cell lines that have been characterized for their ability to promote expression of this foreign gene include the monkey kidney cell, COS-7, and Chinese hamster ovary (CHO) dihydrofolate reductase deficient (dhfr) cells.

3. To establish a continuous CHO-derived cell line that secretes 30kDa TNF inhibitor into cell culture medium, a TNF inhibitor expression plasmid has been introduced into these dhfr cells along with a plasmid that directs the synthesis of dihydrofolate reductase using the calcium phosphate-DNA precipitation technique described by Graham and van der Eb (Virology 52:456-467, 1973). The cells that have taken up DNA and express DHFR were selected as described by Ringold, et al., (J. Mol. Appl. Genet. 1:165-175, 1981).

4. Cells that express the TNF inhibitor gene constructs can be manipulated to increase the levels of production of TNF inhibitor. Cells containing TNF inhibitor expression vectors along with a dhfr expression vector should be taken through the gene amplification protocol described by Ringold, et al., (J. Mol. Appl. Genet. 1:165-175, 1981) using methotrexate, a competitive antagonist of dhfr. Gene amplification leads to more copies of the dhfr and TNF inhibitor genes present in the cells and, concomitantly, increased levels of TNF inhibitor mRNA which, in turn, leads to more TNF inhibitor protein being produced by the cells.

#### Example 10. Isolation of two types of TNF-inhibitors from U937 conditioned medium and the existence of the second TNF inhibitor in human urine.

The human U937 cells were grown at a density of  $1 \times 10^5$  cells per ml in 150 cm<sup>2</sup> flasks using RPMI1640 medium containing 200 units/ml penicillin, 200 units/ml of streptomycin, 10% fetal calf serum. After 3 days of incubation at 37°C the cells were harvested by centrifugation at 1500 G for 7 minutes. The cells were resuspended at a density of  $2 \times 10^6$ /ml in RPMI1640 medium without serum. The cells were grown in the presence of 5 ug/ml PHA-P (Phytohemagglutinin) and 10 ng/ml PMA (Phorbol 12-myristate 13-acetate) for 24 hours.

The 24 hour medium (4425ml) was collected by centrifugation and concentrated by Amicon YM5 filter to about 100 ml. This material was passed through a TNF-affinity gel (0.7 x 2cm) at a flow rate of 0.1 ml/min and the gel was washed extensively with 50mM Tris-HCl pH 7.5. The bound proteins were eluted with 50 mM NaPO<sub>4</sub>-HCl, pH2.5 and TNF inhibitor was separated from other contaminating proteins by HPLC-RPC8. As seen in Figure 27 two TNF-inhibitor peaks are observed. SDS-PAGE analysis of the RPC8 fractions shows that the molecular weights of the two peaks correspond roughly to 30kDa and 40kDa proteins (Figure 25). The 30kDa protein (TNF-1NH1) was subjected to amino-terminal sequence analysis, and found to be the same sequence as that of urinary 30kDa TNF-inhibitor described above. However, the protein sequence of the 40kDa protein reveals that it is not the same as the 30kDa protein (see Example 11). Further purification of the second TNF inhibitor peak in the human urine, which is seen around fraction 35 in Figure 3, shows that it is also the 40kDa TNF-inhibitor protein (Figures 29 and 30).

The 40kDa TNF inhibitor is also a glycoprotein. This was detected using Concanavalin A-peroxidase after the

protein was transferred onto a nitrocellulose filter as described in Example 1 D. The molecular weight of N-glycanase treated 40kDa TNF inhibitor was shown on SDS-PAGE to be about 36kDa. (See procedure described Example 1 D.)

Following the procedures as outlined in Example 1 E, above, it may be determined that the deglycosylated 40kDa TNF inhibitor also binds to TNF alpha. In addition, the deglycosylated 40kDa protein may also be shown to bind to TNF beta (lymphotoxin).

Example 11. Protein sequencing of U937 derived 30kDa TNF inhibitor, 40kDa TNF inhibitor, and Urinary 40kDa TNF inhibitor

Amino terminal sequence of the proteins were determined using Applied Biosystem Protein Sequencer, Model 470. Both native and reduced-carboxymethylated proteins were sequenced. Approximately 200 pmoles of reverse phase (RP-8) purified TNF inhibitors were applied to a polybrene filter and subjected to automated Edman degradation. The resulting sequence is shown in Figure 31. It can be seen that the U937-derived 30kDa protein is the same as that formed and identified in urine. The 40kDa TNF inhibitor protein is not same as the 30kDa TNF inhibitor protein. The urinary 40kDa TNF inhibitor protein does not contain two amino terminal residues; otherwise, it is same as that of the U937-derived 40kDa protein.

Example 12. Primary structure of the 40kDa TNF inhibitor

About 40 ug of the reduced and carboxymethylated TNF inhibitor (40kDa) was digested with endoprotease V8 as described above, and the resulting peptides were separated on an RPC18 column (Figure 33). The peptides purified were sequenced using an Applied Biosystem Protein Sequencer, Model 470.

About 90 ug of the reduced and carboxymethylated TNF inhibitor was treated with 5 ug of endopeptidase Arg-C in 0.2M ammonium bicarbonate at 37°C. After 24 hours of digestion, the Arg-C digested material was loaded onto an HPLC-RP8 column to separate peptides (Figure 32). Purified peptides were sequenced as before. Some of the peptides were further digested with TPCK-trypsin or chymotrypsin. About 500 pmole of arg-C16 peptide was treated with 3 ug of TPCK-trypsin (Boehringer Mannheim) in 0.2M ammonium bicarbonate at 37°C for 7 hours, and peptides were separated using RP8 (Figure 34). About 200 pmole of the peptide arg-C10 was digested with one ug of chymotrypsin (Boehringer Mannheim) at 37°C for three and a half hours, and the resulting peptides were separated on an RPC18 (Figure 35).

A partial structure of the TNF inhibitor (40kDa) was determined by aligning various overlapping peptides (Figure 36). A complete primary structure of the 40kDa TNF inhibitor is shown in Figure 38. Residues not identified by protein sequencing were deduced by review of the sequence of the cDNA clone that encodes the 40kDa TNF inhibitor and that is discussed in Example 14A and described in Figure 39.

Example 13. Identification of cDNA clones for the 40kDa TNFa inhibitor

The information presented in Example 9 shows that U937 cells treated with PMA and PHA produce a TNFa inhibitor with a molecular weight of approximately 40kDa. This protein has been purified and its amino acid sequence has been substantially determined, as described in Example 12. Table 5 shows the sequences of several peptides derived from this protein and gives the sequences of mixed sequence oligonucleotide probes used to isolate genes coding for the 40kDa TNF inhibitor described here.

The gene encoding sequences comprising the 40kDa inhibitor may be isolated from the human genomic library described in Example 5, or a cDNA library constructed from mRNA obtained from U937 cells that had been treated with PMA and PHA for about 9 hours (See Example 14). Each library should contain approximately  $1.0 \times 10^6$  recombinant.

TABLE 5

| Peptide Sequence | Probe Name | Probe Sequence                                |
|------------------|------------|---|
| EYYDQTA          | 40KD-P2'   | 5'GAATATTATGATCAAACAGC 3'<br>G C C C G G<br>T |
| AQUAFT           | 40KD-P1    | 5'GTAAAACGAACTTGAGC 3'<br>G G G C G<br>T T T  |
| KQEGCR           | 40KD-PG    | 5'AAACAAGAAGGATGTCG 3'<br>G G G G CAC<br>T    |
| QMCCSKC          | 40KD-P5    | 5'CATTTAGAACAACACATTG 3'<br>C GCTG G C<br>T   |
| DQTAQMC          | 40KD-P6'   | 5'GATCAAACAGCACAAATGTG 3'<br>C G G G G<br>T T |
| PGWYCA           | 40KDP7     | 5'CCAGGATGGTATTGTGC 3'<br>G G<br>T T          |

#### Example 14. Isolation of 40kDa TNF inhibitor cDNA sequences from PMA/PHA-induced U937 cells

U937 mRNA was isolated from cells that had been induced by PMA/PHA for 9 hours. It was then selected on an oligo-dT column, and the polyadenylated mRNA thus isolated was used to make cDNA using reverse transcriptase followed by *E. coli* polymerase I/RNase H. The cDNA was subjected to a polymerase chain reaction using, as primers, the degenerate probes (40KD-P1' and 40KD-P7) shown in Table 5. The DNA products from this reaction were probed on a Southern blot with probe 40KD-P6' (see Table 5) identifying a single band that contained this sequence. This band was isolated on an agarose gel and cloned into M13 phage DNA (strain mp18). After transformation into *E. coli* strain JM109 and plating on medium containing X-gal and IPTG, clear plaques were identified that contained the correct cDNA insert. The sequence of the DNA in this clone is shown in Figure 37 along with the translation product predicted from this sequence. This amino acid sequence matches the peptide sequence shown in Figure 36 (residues 12 - 104) and Figure 38.

#### Example 14A Isolation of 40kDa TNF inhibitor cDNA clone from PMA/PHA-induced U937 Cells

mRNA was isolated (Chirgwin, J.M. et al. Biochemistry 18, 5294-5299) from human U937 cells that had been exposed to PHA and PMA for 9 hours. mRNA was purified from this RNA using oligo-dT cellulose (Aviv, H. and Leder P. 1972 Proc. Natl. Acad. Sci. (USA) 69, 1408-1412). 5 ug of this mRNA was used to synthesize 3 ug of blunt-ended double-stranded cDNA (Gubler U. and Hoffman B.J. 1983. Gene 25, 263-269). After addition of *Eco*RI linkers the

cDNA was purified by sephacryl S-400 (Pharmacia) spun column chromatography and ethanol precipitated. One hundred ng of this cDNA was ligated into 1 µg of *Eco*RI-digested and alkaline phosphatase-treated lambda gt-10 and packaged *in vitro* using giga-pack gold (Stratagene). The packaged cDNA yielded  $2.5 \times 10^6$  recombinants when plated on *E. coli* C600 hfl.  $1.2 \times 10^6$  members of this library were screened in duplicate with  $^{32}$ P-labeled probe 40KD-P6-7 (5' GGG CGT ATG TGC TGT CCT CAC AGG 3') as described (Benton, W.D. and Davis, R.W. 1977. Science 196:130-132). Twelve positive hybridizing clones were isolated and rescreened with probes 40KD-P6 and 40KD-P7 (see Table 5 in Example 13). Four of these clones hybridized to all three probes. One of these clones, c40DK#6, was digested with *Eco*RI and a 2.2 kb insert was isolated and subcloned in both orientations into the bacteriophage M13 vector mp19 (Yarrish-Perron, C. et al., 1985. Gene 33: 103-119). The sequence was determined from both strands using the chain termination method (Sanger, F. and Coulson, A.R. 1975. J. Mol. Biol. 94: 441-448) with Taq DNA polymerase (U.S. Biochemical). This sequence is shown in Figure 39 along with its deduced translation product. The sequence contains a single open reading frame extending from the ATG triplet at base 93 that extends well beyond the C-terminal sequence of the 40kDa protein at the GAC triplet at base 863.

#### Example 15. The 40kDa TNF inhibitor inhibits TNF beta as well as TNF alpha

Both the 30kDa TNF inhibitor and the 40kDa TNF inhibitor were examined to determine if they were also capable of inhibiting the activity of TNF beta (lymphotoxin). Various concentrations of TNF-beta (purchased from Endogen) were incubated with each of the inhibitors for one hour at room temperature. The resultant mixtures were analyzed via the L929 cell assay system as described in Example 1 B.1. for TNF alpha. These experiments revealed that the 30kDa TNF inhibitor had little inhibitory effect on TNF beta. However, the 40kDa TNF inhibitor showed significant TNF beta inhibition. The results of these experiments can be seen in Figure 40.

#### Example 16. Preparation of human genomic DNA library for 40kDa inhibitor

An appropriate human genomic DNA library for 40kDa TNF inhibitor may be performed as described in Example 5 for 30kDa TNF inhibitor.

#### Example 17. Preparation of genes for the Expression of the 40kDa TNF inhibitor cDNA in *Escherichia coli*

Portions of the TNF inhibitor (40 kDa) cDNA gene coding for soluble TNF binding activities (Fig. 39) have been prepared for expression in *E. coli* as described below.

Because it has been difficult to definitively determine the C-terminal sequence of the mature 40kDa TNF inhibitor derived from urine or U937 cells, we constructed 3 derivatives of its cDNA coding sequence based on sequence analysis of the cDNA clone. The first extends to the putative transmembrane sequence of this protein base pair 863 (Figure 39) and ends with the peptide sequence Gly Ser Thr Gly Asp. The next two are 51 (Δ51) and 53 (Δ53) amino acids shorter than this clone and end at base pair 710 Ser Pro Thr, and base pair 704 Ser Thr Ser, respectively.

Each of these three C-termini were created by *in vitro* mutagenesis ("MutaGene" BioRad, Richmond, CA) of M13 clones of the cDNA of the 40 kDa TNFα inhibitor. The longest clone was created first by use of the following synthetic oligonucleotides:

1. 5' CAC TGG CGA CTA AGC TTC GCT CTT C 3'

2. 5' GCG GCG CAC GCC GGA TCC GAT CTT GGA GGA TGA TTA  
AAT GTT GCC CGC CCA G 3'

Oligonucleotide 1 inserts a translation termination codon after amino acid 235, Asp, and creates a *Hind*III restriction endonuclease recognition site at that point. Oligonucleotide 2 adapts the N-Terminal sequence of the mature protein Leu Pro Ala bp 159 (Figure 39) for expression in *E. coli* by 1) inserting a Met, ATG codon at amino acid position 1 and 2) inserting a translational coupler sequence and 5' *Bam*HI restriction endonuclease recognition site. The mutagenized fragment was removed by *Bam*HI/*Hind*III digestion of Rf DNA of the mutant M13 clone and inserted into an *E. coli* expression plasmid as described in Example 7. Clones bearing this gene construction are called TNF 40.

The two shortened clones were constructed as above using the mutagenized M13 derivative of the 40kDa TNFα inhibitor clone isolated above and the following oligonucleotides.

5' GTCCCCCACCTAAGCTTCGGAGTATGG 3'  $\Delta 51$

5' GTCCACGTCCTAAGCTTCCCACCCGGA 3'  $\Delta 53$

These two oligonucleotides introduce translation termination codons at bp 710 and 704 respectively (Figure 39). Clones bearing these gene constructions are called TNF 40 51 and TNF 40 53 respectively.

10 Example 18. Expression of genes encoding 40kDa TNF inhibitor in animal cells

Expression of the 40kDa TNF inhibitor clone in animal cells may be performed as described in Example 9. The extensive region located 3' of the c-terminus of the 40kDa TNF inhibitor may be deleted and a stop codon engineered into the position just following the c-terminal Aspartic acid.

15

Example 19. Expression of the complete cDNA encoding 30kDa TNF inhibitor in mammalian cells increases TNF receptor sites

20 An expression vector was made that incorporated the entire 30kDa TNF inhibitor cDNA (2.1 kb) shown in Figure 21, named p30KXVA, and was in all other respects identical to the vector shown in Figure 23 (i.e., the TNF-BP sequences shown in that figure were replaced by the 2.1 kb cDNA using the unique EcoRI site in the plasmid). See Example 9 for a more complete description of the expression vector. This plasmid was introduced into COS7 cells using the lipofection procedure described by Feigner *et al.* (Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)). Transfected cells were analyzed for their ability to bind [<sup>125</sup>I]TNF $\alpha$ . Figure 41 shows the results of the binding assay of cells that were mock-transfected or transfected with the expression vector p30KXVA. The number of binding sites on plasmid-transfected cells is dramatically higher than the number on the control cells. The complete cDNA clone (i.e., the open reading frame that encodes a much larger protein than the 30kDa urine-derived inhibitor), in fact, represents a cDNA clone of a TNF receptor.

30 Example 20. Expression of cDNA encoding 40KDa TNF inhibitor in mammalian cells increases TNF receptor sites

35 An expression vector was made using the 2.4 kb cDNA fragment isolated from the lambda phage page #6 described in Example 14A. This plasmid was identical to that described in Example 9 (Figure 23) except that the 40kDa TNF inhibitor cDNA sequences were substituted for the 30kDa TNF inhibitor cDNA sequences in that plasmid. Plasmids were isolated with the 2.4 kb EcoRI cDNA fragment in each orientation, named p40KXVA (sense orientation) and p40KXVB (anti-sense orientation). These plasmids contain the SV40 origin of replication, the cytomegalovirus immediate early promoter and enhancer, the rabbit B-globin second intron, the 40KDa TNF inhibitor cDNA, and the SV50 early polyadenylation signal (for a more complete description of this vector, see Example 9) in a pBR322-based plasmid. These plasmids were transfected into COS7 cells which were then assayed for TNF binding (see Figure 42). Cells transfected with p40KXVA exhibited a higher number of TNF binding sites on the cell surface than either COS7 cells alone or COS7 cells transfected with p40KXVB, suggesting that this cDNA encodes a TNF receptor. Other mammalian cells such as CHO cells could be developed that could overproduce this receptor or that secrete 40KDa TNF inhibitor into the tissue culture medium in ways described in Example 9.

45 Example 21. Inhibitor isolated from human monocytes

50 Human monocytes were prepared from 550 ml of blood as described by (Hannum, C. H. *et al.* Nature 343, 336-340, 1990). The fresh monocytes ( $2 \times 10^7$  cells) were seeded in 500 ml of serum free RPMI1640 medium and treated with 10ng/ml of PMA and 5ug/ml of PHA-P for 24, 48 and 72 hours at 37°C. After the incubation, the media were collected by centrifugation and concentrated to 50 ml. The concentrated media were loaded onto a TNF-affinity column (2 ml bed volume) one sample at a time and eluted with acid as in Example 1. The eluted material was further purified using a HPLC RPC-8 column under the same conditions as in Example 1, and each fraction was assayed with L929 cytotoxicity assay. Figure 43 shows the two peaks of TNF inhibition activity. These two peaks correspond to the 30 kDa and 40 kDa TNF inhibitors which were also found in the culture medium of U937 cells that was treated with PMA and PHA and identified in urine.

55

Example 22. Expression and Purification of shorter forms of the 40 kDa TNF inhibitor ( $\Delta$ 51 and  $\Delta$ 53) from E. coli

Cells 300 ml of E. coli cultures (40kDa TNF inhibitor  $\Delta$ 51 and 40kDa TNF inhibitor  $\Delta$ 53) grown separately under induced condition for 2 hours were resuspended in 10 ml of 50 mM Tris-HCl, pH 7.5 containing 2mM EDTA (TE buffer) and French pressed at 20 000G for 10 min. The resulting pellets were washed once with TE buffer. The washed pellet was resuspended in 2 ml of 6M Guanidine-HCl/100mM Tris-HCl, pH8.5/4mM PMSF and incubated at room temperature for one hour. After incubation 500mM DTT was added to a final concentration of 4 mM, and the mixture was incubated at room temperature for another hour. Insoluble material was removed by centrifugation at 20 000G for 15 min. 500mM oxidized glutathione was added to the supernatant to a final concentration of 20 mM, and the mixture was incubated at room temperature for 10 min. This material was then diluted in 20 ml of 0.6% Tris base solution with 5 mM cysteine. PMSF was added to a final concentration of 2mM. After 16 hours of incubation at 4°C, this material was dialyzed against 300 volumes of 50mM Tris-HCl, pH 7.5 for 3 hours at 4°C, then centrifuged at 20,000G for 15 min. The supernatant was loaded onto a TNF-affinity column (7 x 2 cm, 13 mg rhTNF/ml of affigel-10) at a flow rate of 0.09 ml per min. This column was extensively washed with 50mM Tris-HCl, pH 7.5. The bound proteins were eluted with 50mM NaH<sub>2</sub>PO<sub>4</sub>-HCl, pH 2.5. The acidic eluates were loaded onto an RP8 column (2 x 200mm, spelco) and the TNF inhibitors were eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml per gradient per min. (Figure 44A and 45A). Fractions were examined by L929 cytotoxicity assay to localize the TNF inhibitors. The major peak on each RP8 profile contains the TNF-inhibiting activity (Figure 44B and 45B). The E. coli-produced TNF-inhibitors (40kDa TNF inhibitor  $\Delta$ 53 and 40kDa TNF inhibitor  $\Delta$ 51) migrate to the expected location on SDS-PAGE (Figure 44B and 45B). The amino terminal sequence of these materials shows that the E. coli-produced TNF-inhibitors have the following sequence:

**Met-Leu-Pro-Ala-Gln-Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu**

By using this procedure, about 150ug of each 40kDa TNF inhibitor ( $\Delta$ 51 and  $\Delta$ 53) was obtained from 30 ml of the culture. The yield was a few percent, however, the yield can be increased to over 30% by improving each step of this purification.

Both of these 40kDa TNF inhibitors ( $\Delta$ 51 and  $\Delta$ 53) inhibit not only TNF-alpha but also TNF-beta.

Example 23. Expression and Purification of full length 40 kDa TNF inhibitor.

An active 40kDa TNF inhibitor was purified from an E. coli strain carrying plasmids which have a gene for full length mature 40 kDa TNF inhibitor (as in Example 12). The method used to isolate an active inhibitor was the same as that of example 22. This active inhibitor inhibits both TNF-alpha and TNF-beta, and the amino terminal sequence is same as shown in Example 22.

Example 24. Amino acid composition of the 40 kDa TNF Inhibitor.

U937-produced mature 40 kDa TNF inhibitor was analyzed for total amino acid composition by the PTC-amino acid analysis system. The actual and predicted composition data for full length mature 40kDa TNF inhibitor as shown in Figure 38 are shown in Table 6.

Example 25. Production of chemically modified TNF inhibitors.

In order to increase the half-life of the TNF inhibitors in plasma, TNF inhibitors which are chemically modified with polyethylene glycol (PEG) may be made. The modification may be done by cross linking PEG to a cysteine residue of the TNF inhibitor molecules. Since all of the cysteine residues in the TNF inhibitors form disulfide bonds, mutant TNF inhibitors may be constructed which contain an extra cysteine residue at the amino terminus, glycosylation sites, and the carboxyl terminus of each inhibitor. The mutagenesis may be carried out by PCR using oligonucleotides containing the desired mutation. As for the 30kDa TNF inhibitor, an extra cysteine residue was added at residue number 1, 14 or 105. These mutant proteins were expressed in E. coli by using the same system described in Examples 7, 22 and 23, and refolded to active TNF inhibitor. The mutant proteins are as active as the non-mutated proteins. Pegylation of these proteins will be carried out, and the activity will be assessed. The 40 kDa mutants will be constructed as above and pegylation will be performed to obtain active proteins and will have increased the stability of the TNF inhibitor.



TABLE 6

|                     | Calculated # by DNA sequence | Experimental # |
|---------------------|------------------------------|----------------|
| Asx                 | 14                           | 13.0           |
| Glx                 | 23                           | 22.6           |
| Ser                 | 25                           | 23.2           |
| Gly                 | 14                           | 17.3           |
| His                 | 4                            | 4.5            |
| Thr                 | 26                           | 23.9           |
| Ala                 | 17                           | 17             |
| Arg                 | 14                           | 15.1           |
| Pro                 | 26                           | 22.3           |
| Val                 | 13                           | 3.7            |
| Ile                 | 4                            | 3.4            |
| Leu                 | 10                           | 3.6            |
| Phe                 | 5                            | 4.6            |
| Lys                 | 6                            | 5.4            |
| Tyr                 | 5                            | 5.0            |
| Trp                 | 3                            | ND             |
| Met                 | 3                            | ND             |
| Cys                 | 22                           | ND             |
| ND: not determined. |                              |                |

It is to be understood that the application of the teachings of the present invention to a specific expression system will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Thus, it will be apparent to those of ordinary skill in the art that various modifications and variations can be made in the processes and products of the present invention. It is intended that the present invention covers these modifications and variations provided they come within the scope of the appended claims and their equivalents.

### Claims

Claims for the following Contracting States : AT, BE, CH, DE, DK, FR, GB, IT, LI, LU, NL, SE

1. A tumor necrosis factor (TNF) inhibitor comprising the amino acid sequence shown in Figure 19 wherein said inhibitor is produced in a host cell which is not capable of glycosylation or which is a non-human cell capable of glycosylation.
2. A tumor necrosis factor (TNF) inhibitor which is non-glycosylated and has a molecular weight of about 19 kDa.
3. The TNF inhibitor of claim 2, wherein said TNF inhibitor is produced by recombinant-DNA methods.
4. A TNF inhibitor comprising the amino acid sequence as shown by residues 1 through 235 in Fig. 38 (40 kDa inhibitor), as shown by residues 1 through 182 in Figure 38 (40 kDa inhibitor Δ53), or as shown by residues 1 through 124 in Figure 38 (40 kDa inhibitor Δ51).
5. The TNF inhibitor of claim 4, wherein said TNF inhibitor is produced in a host cell which is not capable of glycosylation or which is a non-human cell capable of glycosylation.
6. A TNF inhibitor comprising the amino acid sequence as set forth in Figures 19 or 38, except that the amino acid at the N-terminus, at the C-terminus or at a site of glycosylation is replaced by a cysteine residue.
7. The TNF inhibitor of claim 6, wherein said TNF inhibitor has the amino acid sequence as shown in Figure 19 and said non-naturally-occurring cysteine is at residue number 1, 14 or 105.

8. The TNF inhibitor of any of claims 1 to 3 and 6 wherein said TNF inhibitor is linked to a high molecular weight polymeric material
9. The TNF inhibitor of claim 8, wherein said high molecular weight polymeric material is polyethylene glycol
10. A pharmaceutical preparation comprising the TNF inhibitor according to any one of claims 1 to 9 in a pharmaceutically acceptable carrier
11. A method for the production of a TNF inhibitor comprising:
  - (a) culturing under conditions suitable for expression of said TNF inhibitor a host cell containing a nucleic acid comprising a sequence selected from the group consisting of:
    - (i) the DNA sequence as shown in Fig. 13 or a coding portion thereof;
    - (ii) the DNA sequence as shown in Fig. 20 or a coding portion thereof;
    - (iii) the DNA sequence as shown in Fig. 21 or a coding portion thereof;
    - (iv) the DNA sequence as shown in Fig. 37 or a coding portion thereof;
    - (v) the DNA sequence as shown in Fig. 39 or a coding portion thereof;
    - (vi) a sequence which is degenerate in the coding regions or portions thereof of (i), (ii), (iii), (iv) and (v);
    - (vii) a sequence which hybridizes to a nucleic acid sequence which is complementary to (i), (ii), (iii), (iv) (v) or (vi).
  - (b) harvesting said inhibitor.
12. The method of claim 11, further comprising before step (a) the steps:
  - I) Preparation of a nucleic acid comprising a sequence selected from the group consisting of:
    - (i) the DNA sequence as shown in Fig. 13 or a coding portion thereof;
    - (ii) the DNA sequence as shown in Fig. 20 or a coding portion thereof;
    - (iii) the DNA sequence as shown in Fig. 21 or a coding portion thereof;
    - (iv) the DNA sequence as shown in Fig. 37 or a coding portion thereof;
    - (v) the DNA sequence as shown in Fig. 39 or a coding portion thereof;
    - (vi) a sequence which is degenerate in the coding regions or portions thereof of (i), (ii), (iii), (iv) and (v);
    - (vii) a sequence which hybridizes to a nucleic acid sequence which is complementary to (i), (ii), (iii), (iv), (v) or (vi).
  - II) Cloning the DNA sequence into a vector capable of being transferred into and replicated in a whole cell such vector containing operational elements needed to express the DNA sequence;
  - III) Transferring the vector containing the synthetic DNA sequence and operational elements into a host capable of expressing the DNA encoding the TNF inhibitor.
13. The method of claim 11, further comprising the step:
  - (c) permitting the inhibitor to assume an active tertiary structure whereby it possesses TNF inhibitory activity.
14. The method of claim 11, wherein said TNF inhibitor comprises the amino acid sequence shown by:
  - (i) Figure 19;
  - (ii) Figure 38;
  - (iii) residues 1 through 184 as set forth in Figure 38 (40kDa inhibitor  $\Delta$ 51); or
  - (iv) residues 1 through 182 as set forth in Figure 38 (40kDa inhibitor  $\Delta$ 53)
15. The method of claim 11, wherein said TNF inhibitor has a non-naturally occurring cysteine residue at the C-terminus, the N-terminus, or a glycosylation site of said inhibitor
16. The method of claim 15, wherein said TNF inhibitor comprises the amino acid sequence as shown in Figure 19 and said non-naturally occurring cysteine is at the N-terminus, C-terminus or at residue numbers 1, 14 or 105

17. A nucleic acid comprising a sequence selected from the group consisting of:

- (i) the DNA sequence as shown in Fig. 13;
- (ii) the DNA sequence as shown in Fig. 20;
- 5 (iii) the DNA sequence as shown in Fig. 21;
- (iv) the DNA sequence as shown in Fig. 37;
- (v) the DNA sequence as shown in Fig. 39;
- (vi) a fragment of (i), (ii), (iii), (iv) or (v) encoding a polypeptide having TNF inhibitory activity;
- (vii) a sequence which is degenerate of (i), (ii), (iii), (iv), (v) or (vi) encoding a polypeptide having TNF inhibitory activity;
- 10 (viii) a sequence which is complementary to (i), (ii), (iii), (iv), (v), (vi) or (vii);
- (ix) a sequence which hybridizes to (viii) and which encodes a polypeptide having TNF inhibitory activity.

18. The TNF inhibitor according to any of claims 1 to 9 for use in the treatment of a TNF-mediated disease

19. The TNF inhibitor according to any of claims 1 to 9 for use in a treatment of a TNF-mediated disease in combination with an IL-1 inhibitor.

20. Use of the TNF inhibitor according to any of claims 1 to 9 for the preparation of a medicament for treating or preventing TNF mediated diseases.

21. The use according to claim 20, wherein said medicament further comprises an IL-1 inhibitor.

22. A vector comprising a nucleic acid sequence as defined in claim 17.

23. A host cell containing a recombinant DNA molecule comprising a nucleic acid sequence as defined in claim 17

24. The host cell according to claim 23, which is a prokaryotic cell.

25. The host cell according to claim 24 which is an *E. coli*.

26. The host cell according to claim 23, which is a eukaryotic cell.

27. The host cell according to claim 26, wherein said eukaryotic cell is a mammalian cell.

28. The host cell according to claim 27, wherein said mammalian cell is a Chinese hamster ovary cell.

#### Claims for the following Contracting State : ES

1. A method for the production of a TNF inhibitor comprising:

(a) culturing under conditions suitable for expression of said TNF inhibitor a host cell containing a nucleic acid comprising a sequence selected from the group consisting of:

- (i) the DNA sequence as shown in Fig. 13 or a coding portion thereof;
- (ii) the DNA sequence as shown in Fig. 20 or a coding portion thereof;
- (iii) the DNA sequence as shown in Fig. 21 or a coding portion thereof;
- (iv) the DNA sequence as shown in Fig. 37 or a coding portion thereof;
- (v) the DNA sequence as shown in Fig. 39 or a coding portion thereof;
- (vi) a sequence which is degenerate in the coding regions or portions thereof of (i), (ii), (iii), (iv) and (v);
- (vii) a sequence which hybridizes to a nucleic acid sequence which is complementary to (i), (ii), (iii), (iv), (v) or (vi);

(b) harvesting said inhibitor.

2. The method of claim 1 further comprising before step (a) the steps:

I) Preparation of a nucleic acid comprising a sequence selected from the group consisting of

- (i) the DNA sequence as shown in Fig. 13 or a coding portion thereof;
- (ii) the DNA sequence as shown in Fig. 20 or a coding portion thereof;
- (iii) the DNA sequence as shown in Fig. 21 or a coding portion thereof;
- (iv) the DNA sequence as shown in Fig. 37 or a coding portion thereof;
- (v) the DNA sequence as shown in Fig. 39 or a coding portion thereof;
- (vi) a sequence which is degenerate in the coding regions or portions thereof of (i), (ii), (iii), (iv) and (v);
- (vii) a sequence which hybridizes to a nucleic acid sequence which is complementary to (i), (ii), (iii), (iv), (v) or (vi).

II) Cloning the DNA sequence into a vector capable of being transferred into and replicated in a whole cell, such vector containing operational elements needed to express the DNA sequence;

III) Transferring the vector containing the synthetic DNA sequence and operational elements into a host capable of expressing the DNA encoding the TNF inhibitor.

3. The method of claim 1 further comprising the step.

(c) permitting the inhibitor to assume an active tertiary structure whereby it possesses TNF inhibitory activity.

4. The method of claim 1, wherein said TNF inhibitor has the amino acid sequence shown by:

- (i) Figure 19;
- (ii) Figure 38;
- (iii) residues 1 through 184 as set forth in Figure 38 (40 kDa inhibitor  $\Delta$ 51); or
- (iv) residues 1 through 182 as set forth in Figure 38 (40 kDa inhibitor  $\Delta$ 53).

5. The method of claim 1, wherein said TNF inhibitor has a non-naturally occurring cysteine residue at the C-terminus, the N-terminus, or a glycosylation site of said inhibitor.

6. The method of claim 5, wherein said TNF inhibitor comprises the amino acid sequence as shown in Figure 19 and said non-naturally occurring cysteine is at the N-terminus, C-terminus or at residue numbers 1-14 or 105.

7. The method of claim 6, wherein said TNF inhibitor is linked to a high molecular weight polymeric material.

8. The method of claim 7, wherein said high molecular weight polymeric material is polyethylene glycol.

9. A method for preparing a pharmaceutical preparation comprising the TNF inhibitor, wherein the TNF inhibitor produced according to any of claims 1 to 8 is combined with a pharmaceutically acceptable carrier in a manner known per se.

10. A method for preparing a nucleic acid, wherein a nucleic acid comprising a sequence selected from the group consisting of:

- (i) the DNA sequence as shown in Fig. 13;
- (ii) the DNA sequence as shown in Fig. 20;
- (iii) the DNA sequence as shown in Fig. 21;
- (iv) the DNA sequence as shown in Fig. 37;
- (v) the DNA sequence as shown in Fig. 39;
- (vi) a fragment of (i), (ii), (iii), (iv) or (v) encoding a polypeptide having TNF inhibitory activity;
- (vii) a sequence which is degenerate of (i), (ii), (iii), (iv), (v) or (vi) encoding a polypeptide having TNF inhibitory activity;
- (viii) a sequence which is complementary to (i), (ii), (iii), (iv), (v), (vi) or (vii);
- (ix) a sequence which hybridizes to (viii) and which encodes a polypeptide having TNF inhibitory activity

is synthesized in a manner known per se

11. A method for preparing a recombinant vector wherein a nucleic acid sequence as obtainable according to claim

10 is joined with vector DNA in a manner known per se

12. A method for preparing a host cell comprising introducing a DNA sequence to generate a recombinant DNA molecule containing a nucleic acid sequence as obtainable according to claim 10 into a desired host cell.
13. The method according to claim 12, wherein the host cell is a prokaryotic cell.
14. The method according to claim 13, wherein the host cell is an *E. coli*.
15. The method according to claim 12, wherein the host cell is a eukaryotic cell.
16. The method according to claim 15, wherein said eukaryotic cell is a mammalian cell.
17. The method according to claim 16, wherein said mammalian cell is a Chinese hamster ovary cell.

#### Claims for the following Contracting State : GR

1. A tumor necrosis factor (TNF) inhibitor comprising the amino acid sequence shown in Figure 19 wherein said inhibitor is produced in a host cell which is not capable of glycosylation or which is a non-human cell capable of glycosylation.
2. A tumor necrosis factor (TNF) inhibitor which is non-glycosylated and has a molecular weight of about 18 kDa.
3. The TNF inhibitor of claim 2, wherein said TNF inhibitor is produced by recombinant-DNA methods.
4. A TNF inhibitor comprising the amino acid sequence shown in Figure 38, as shown by residues 1 through 235 (40 kDa inhibitor), or by residues 1 through 182 (40 kDa inhibitor  $\Delta$ 53) in Figure 38, or by residues 1 through 124 as set forth in Figure 38 (40 kDa inhibitor  $\Delta$ 51).
5. The TNF inhibitor of claim 4, wherein said TNF inhibitor is produced in a host cell which is not capable of glycosylation or which is a non-human cell capable of glycosylation.
6. A TNF inhibitor comprising the amino acid sequence as set forth in Figures 19 or 38, except that the amino acid at the N-terminus, at the C-terminus or at a site of glycosylation is replaced by a cysteine residue.
7. The TNF inhibitor of claim 6, wherein said TNF inhibitor has the amino acid sequence as shown in Figure 19 and said non-naturally-occurring cysteine is at residue number 1, 14 or 105.
8. The TNF inhibitor of any of claims 1 to 3 and 6, wherein said TNF inhibitor is linked to a high molecular weight polymeric material.
9. The TNF inhibitor of claim 8, wherein said high molecular weight polymeric material is polyethylene glycol.
10. A method for preparing a pharmaceutical preparation comprising combining the TNF inhibitor according to any one of claims 1 to 9 and a pharmaceutically acceptable carrier.
11. A method for the production of a TNF inhibitor comprising:
  - (a) culturing under conditions suitable for expression of said TNF inhibitor a host cell containing a nucleic acid comprising a sequence selected from the group consisting of:
    - (i) the DNA sequence as shown in Fig. 13 or a coding portion thereof;
    - (ii) the DNA sequence as shown in Fig. 20 or a coding portion thereof;
    - (iii) the DNA sequence as shown in Fig. 21 or a coding portion thereof;
    - (iv) the DNA sequence as shown in Fig. 37 or a coding portion thereof;
    - (v) the DNA sequence as shown in Fig. 39 or a coding portion thereof;
    - (vi) a sequence which is degenerate in the coding regions or portions thereof of (i), (ii), (iii), (iv), and (v).

(viii) a sequence which hybridizes to a nucleic acid sequence which is complementary to (i), (ii), (iii), (iv), (v) or (vi)

(b) harvesting said inhibitor

12. The method of claim 11, further comprising before step (a) the steps:

I) Preparation of a nucleic acid comprising a sequence selected from the group consisting of:

- (i) the DNA sequence as shown in Fig. 13 or a coding portion thereof;
- (ii) the DNA sequence as shown in Fig. 20 or a coding portion thereof;
- (iii) the DNA sequence as shown in Fig. 21 or a coding portion thereof;
- (iv) the DNA sequence as shown in Fig. 37 or a coding portion thereof;
- (v) the DNA sequence as shown in Fig. 39 or a coding portion thereof;
- (vi) a sequence which is degenerate in the coding regions or portions thereof of (i), (ii), (iii), (iv) or (v);
- (vii) a sequence which hybridizes to a nucleic acid sequence which is complementary to (i), (ii), (iii), (iv), (v) or (vi);

II) Cloning the DNA sequence into a vector capable of being transferred into and replicated in a whole cell, such vector containing operational elements needed to express the DNA sequence;

III) Transferring the vector containing the synthetic DNA sequence and operational elements into a host capable of expressing the DNA encoding the TNF inhibitor.

13. The method of claim 11, further comprising the step:

(c) permitting the inhibitor to assume an active tertiary structure whereby it possesses TNF inhibitory activity

14. The method of claim 11, wherein said TNF inhibitor comprises the amino acid sequence shown by:

- (i) Figure 19;
- (ii) Figure 38;
- (iii) residues 1 through 184 as set forth in Figure 38 (40 kDa inhibitor  $\Delta$ 51); or
- (iv) residues 1 through 182 as set forth in Figure 38 (40 kDa inhibitor  $\Delta$ 53).

15. The method of claim 11, wherein said TNF inhibitor has a non-naturally occurring cysteine residue at the C-terminus, the N-terminus, or a glycosylation site of said inhibitor.

16. The method of claim 15, wherein said TNF inhibitor comprises the amino acid sequence as shown in Figure 19 and said non-naturally occurring cysteine is at the N-terminus, C-terminus or at residue numbers 1, 14 or 105

17. A nucleic acid comprising a sequence selected from the group consisting of:

- (i) the DNA sequence as shown in Fig. 13;
- (ii) the DNA sequence as shown in Fig. 20;
- (iii) the DNA sequence as shown in Fig. 21;
- (iv) the DNA sequence as shown in Fig. 37;
- (v) the DNA sequence as shown in Fig. 39;
- (vi) a fragment of (i), (ii), (iii), (iv) or (v) encoding a polypeptide having TNF inhibitory activity;
- (vii) a sequence which is degenerate of (i), (ii), (iii), (iv), (v) or (vi) encoding a polypeptide having TNF inhibitors activity;
- (viii) a sequence which is complementary to (i), (ii), (iii), (iv), (v), (vi) or (vii);
- (ix) a sequence which hybridizes to (viii) and which encodes a polypeptide having TNF inhibitory activity.

18. The TNF inhibitor according to any of claims 1 to 9 for use in the treatment of a TNF-mediated disease

19. The TNF inhibitor according to any of claims 1 to 9 for use in the treatment of a TNF-mediated disease in combination with an IL-1 inhibitor

20. Use of the TNF inhibitor according to any of claims 1 to 9 for the preparation of a medicament for treating or preventing TNF-mediated diseases

21. The use according to claim 20 wherein said medicament further comprises an IL-1 inhibitor.

22. A vector comprising a nucleic acid sequence as defined in claim 17.

23. A host cell containing a recombinant DNA molecule comprising a nucleic acid sequence as defined in claim 17

24. The host cell according to claim 23, which is a prokaryotic cell.

25. The host cell according to claim 24 which is an *E. coli*.

26. The host cell according to claim 23, which is a eukaryotic cell.

27. The host cell according to claim 26, wherein said eukaryotic cell is a mammalian cell.

28. The host cell according to claim 27, wherein said mammalian cell is a Chinese hamster ovary cell.

## Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, GB, IT, LI, LU, NL, SE

1. Tumor Nekrose-Faktor (TNF)-Inhibitor, umfassend die in Abbildung 19 dargestellte Aminosäuresequenz, wobei der Inhibitor in einer Wirtszelle hergestellt wird, die keine Glykosylierung durchführen kann oder die eine nicht-menschliche Zelle ist, welche Glykosylierung durchführen kann.

2. Tumor Nekrose-Faktor (TNF)-Inhibitor, der nicht glykosyliert ist und ein Molekulargewicht von circa 18 kDa hat.

3. TNF-Inhibitor nach Anspruch 2, wobei der TNF-Inhibitor durch rekombinante DNA-Verfahren hergestellt wird.

4. TNF-Inhibitor, umfassend die Aminosäuresequenz wie dargestellt in Abbildung 38 von Aminosäure 1 bis 235 (40 kDa-Inhibitor), von Aminosäure 1 bis 182 wie dargestellt in Abbildung 38 (40 kDa-Inhibitor Δ53) oder von Aminosäure 1 bis 184 wie dargestellt in Abbildung 38 (40 kDa-Inhibitor Δ51).

5. TNF-Inhibitor nach Anspruch 4, wobei der TNF-Inhibitor in einer Wirtszelle hergestellt wird, die keine Glykosylierung durchführen kann oder die eine nicht-menschliche Zelle ist, welche Glykosylierung durchführen kann.

6. TNF-Inhibitor, umfassend die Aminosäuresequenz wie dargestellt in Abbildungen 19 oder 38, mit der Ausnahme, daß die Aminosäure am N-Terminus, am C-Terminus oder an einer Glykosylierungsstelle durch einen Cysteinrest ersetzt ist.

7. TNF-Inhibitor nach Anspruch 6, wobei der TNF-Inhibitor eine die in Abbildung 19 dargestellte Aminosäuresequenz hat und das nicht natürlich vorkommende Cystein in Position Nr. 1, 14 oder 105 liegt.

8. TNF-Inhibitor nach einem der Ansprüche 1 bis 3 und 6, wobei der TNF-Inhibitor an ein polymeres Material mit hohem Molekulargewicht gebunden ist.

9. TNF-Inhibitor nach Anspruch 8, wobei das polymere Material mit hohem Molekulargewicht Polyethylenglykol ist.

10. Pharmazeutische Zubereitung, umfassend den TNF-Inhibitor nach einem der Ansprüche 1 bis 9 in einem pharmazeutisch verträglichen Träger.

11. Verfahren zum Herstellen eines TNF-Inhibitors, umfassend:

(a) Kultivieren einer Wirtszelle unter zur Expression des TNF-Inhibitors geeigneten Bedingungen, wobei die

Wirtszelle eine Nukleinsäure enthält, die eine Sequenz umfaßt, die aus der aus

- (i) der in Abbildung 13 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (ii) der in Abbildung 20 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (iii) der in Abbildung 21 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (iv) der in Abbildung 37 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (v) der in Abbildung 39 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (vi) einer Sequenz, die in den kodierenden Bereichen von (i), (ii), (iii), (iv) und (v) oder Teilen davon degeneriert ist;
- und
- (vii) einer Sequenz, die an eine Nukleinsäure-Sequenz hybridisiert, die komplementär zu (i), (ii), (iii), (iv), (v) oder (vi) ist;

bestehenden Gruppe ausgewählt ist;

(b) Ernten des Inhibitors.

12. Verfahren nach Anspruch 11, umfassend vor Schritt (a) weiterhin die Schritte:

I) Herstellen einer Nukleinsäure, die eine Sequenz umfaßt, die aus der aus

- (i) der in Abbildung 13 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (ii) der in Abbildung 20 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (iii) der in Abbildung 21 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (iv) der in Abbildung 37 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (v) der in Abbildung 39 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
- (vi) einer Sequenz, die in den kodierenden Bereichen von (i), (ii), (iii), (iv) und (v) oder Teilen davon degeneriert ist;
- und
- (vii) einer Sequenz, die an eine Nukleinsäure-Sequenz hybridisiert, die komplementär zu (i), (ii), (iii), (iv), (v) oder (vi) ist;

bestehenden Gruppe ausgewählt ist;

II) Klonieren der DNA-Sequenz in einen Vektor, der in eine vollständige Zelle übertragen und repliziert werden kann, wobei dieser Vektor funktionelle Elemente enthält, die zur Expression der DNA-Sequenz benötigt werden;

III) Übertragen des Vektors, der die synthetische DNA-Sequenz und funktionelle Elemente enthält, in einen Wirt, der die DNA, die für den TNF-Inhibitor kodiert, exprimieren kann.

13. Verfahren nach Anspruch 11, weiter umfassend einen Schritt (c), in dem dem Inhibitor gestattet wird, eine aktive Tertiärstruktur anzunehmen, wodurch er TNF-inhibitorische Aktivität besitzt.

14. Verfahren nach Anspruch 11, wobei der TNF-Inhibitor eine Aminosäuresequenz umfaßt, die dargestellt ist in

- (i) Abbildung 19;
- (ii) Abbildung 38;
- (iii) Aminosäuren 1 bis 184, wie in Abbildung 38 (40kDa-Inhibitor Δ51) dargestellt; oder
- (iv) Aminosäuren 1 bis 182, wie in Abbildung 38 (40kDa-Inhibitor Δ53) dargestellt.

15. Verfahren nach Anspruch 11, wobei der TNF-Inhibitor einen nicht natürlich vorkommenden Cysteinrest am C-Terminus, am N-Terminus, oder an einer Glykosylierungsstelle des Inhibitors enthält.

16. Verfahren nach Anspruch 15, wobei der TNF-Inhibitor die in Abbildung 19 dargestellte Aminosäuresequenz umfaßt und das nicht natürlich vorkommende Cystein am N-Terminus, C-Terminus oder den Aminosäurepositionen 1 bis 105 ist.



17. Nukleinsäure umfassend eine Sequenz, die aus der aus

- (i) der in Abbildung 13 dargestellten DNA-Sequenz
  - (ii) der in Abbildung 20 dargestellten DNA-Sequenz
  - (iii) der in Abbildung 21 dargestellten DNA-Sequenz
  - (iv) der in Abbildung 37 dargestellten DNA-Sequenz
  - (v) der in Abbildung 39 dargestellten DNA-Sequenz
  - (vi) einem Fragment von (i), (ii), (iii), (iv) oder (v), das für ein Polypeptid mit TNF-inhibitorischer Aktivität kodiert
  - (vii) einer Sequenz, die im Vergleich zu den Sequenzen von (i), (ii), (iii), (iv), (v) oder (vi) degeneriert ist, die für ein Polypeptid mit TNF-inhibitorischer Aktivität kodiert
  - (viii) einer Sequenz, die zu (i), (ii), (iii), (iv), (v) oder (vi) komplementär ist
  - (ix) einer Sequenz, die mit (viii) hybridisiert und die für ein Polypeptid mit TNF-inhibitorischer Wirkung kodiert
- bestehenden Gruppe ausgewählt ist.

- 18. TNF-Inhibitor nach einem der Ansprüche 1 bis 9 zur Verwendung beim Behandeln einer TNF-vermittelten Krankheit.
- 19. TNF-Inhibitor nach einem der Ansprüche 1 bis 9 zur Verwendung in Kombination mit einem IL-1-Inhibitor beim Behandeln einer TNF-vermittelten Krankheit.
- 20. Verwendung des TNF-Inhibitors nach einem der Ansprüche 1 bis 9 zum Herstellen eines Medikamentes zum Behandeln oder Vorbeugen von TNF-vermittelten Krankheiten
- 21. Verwendung nach Anspruch 20, wobei das Medikament weiterhin einen IL-1-Inhibitor umfaßt.
- 22. Vektor, umfassend eine Nukleinsäure-Sequenz wie in Anspruch 17 definiert.
- 23. Wirtszelle, enthaltend ein rekombinantes DNA-Molekül, umfassend eine Nukleinsäure-Sequenz wie in Anspruch 17 definiert.
- 24. Wirtszelle nach Anspruch 23, die eine prokaryotische Zelle ist.
- 25. Wirtszelle nach Anspruch 24, die ein *E. coli* ist.
- 26. Wirtszelle nach Anspruch 23, die eine eukaryotische Zelle ist.
- 27. Wirtszelle nach Anspruch 26, wobei die eukaryotische Zelle eine Säugerzelle ist.
- 28. Wirtszelle nach Anspruch 27, wobei die Wirtszelle eine Chinese hamster ovary-Zelle ist.

#### Patentansprüche für folgenden Vertragsstaat : ES

- 1. Verfahren zum Herstellen eines TNF-Inhibitors, umfassend:
  - (a) Kultivieren einer Wirtszelle unter zur Expression des TNF-Inhibitors geeigneten Bedingungen, wobei die Wirtszelle eine Nukleinsäure enthält, die eine Sequenz umfaßt, die aus der aus:
    - (i) der in Abbildung 13 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
    - (ii) der in Abbildung 20 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
    - (iii) der in Abbildung 21 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
    - (iv) der in Abbildung 37 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
    - (v) der in Abbildung 39 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;
    - (vi) einer Sequenz, die in den kodierenden Bereichen von (i), (ii), (iii), (iv) und (v) oder Teilen davon degeneriert ist;
    - und
    - (vii) einer Sequenz, die an eine Nukleinsäure-Sequenz hybridisiert, die komplementär zu (i), (ii), (iii), (iv)

(v) oder (vi) ist:

bestehenden Gruppe ausgewählt ist.

5 (b) Ernten des Inhibitors.

2. Verfahren nach Anspruch 1, umfassend vor Schritt (a) weiterhin die Schritte

I) Herstellen einer Nukleinsäure, die eine Sequenz umfaßt, die aus der aus:

10

(i) der in Abbildung 13 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;

(ii) der in Abbildung 20 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;

(iii) der in Abbildung 21 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;

(iv) der in Abbildung 37 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;

15

(v) der in Abbildung 39 dargestellten DNA-Sequenz oder einem kodierenden Teil davon;

(vi) einer Sequenz, die in den kodierenden Bereichen von (i), (ii), (iii), (iv) und (v) oder Teilen davon degeneriert ist;

und

(vii) einer Sequenz, die an eine Nukleinsäure-Sequenz hybridisiert, die komplementär zu (i), (ii), (iii), (iv),

20

(v) oder (vi) ist:

bestehenden Gruppe ausgewählt ist:

II) Klonieren der DNA-Sequenz in einen Vektor, der in eine vollständige Zelle übertragen und repliziert werden kann, wobei dieser Vektor funktionelle Elemente enthält, die zur Expression der DNA-Sequenz benötigt werden;

25

III) Übertragen des Vektors, der die synthetische DNA-Sequenz und funktionelle Elemente enthält, in einen Wirt, der die DNA, die für den TNF-Inhibitor kodiert, exprimieren kann.

30

3. Verfahren nach Anspruch 1, weiter umfassend einen Schritt (c), in dem dem Inhibitor gestattet wird, eine aktive Tertiärstruktur anzunehmen, wodurch er TNF-inhibitorische Aktivität besitzt.

4. Verfahren nach Anspruch 1, wobei der TNF-Inhibitor eine Aminosäuresequenz umfaßt, die dargestellt ist in:

35

(i) Abbildung 19;

(ii) Abbildung 38;

(iii) Aminosäuren 1 bis 184, wie in Abbildung 38 (40kDa-Inhibitor  $\Delta 51$ ) dargestellt, oder

(iv) Aminosäuren 1 bis 182, wie in Abbildung 38 (40kDa-Inhibitor  $\Delta 53$ ) dargestellt.

40

5. Verfahren nach Anspruch 1, wobei der TNF-Inhibitor einen nicht natürlich vorkommenden Cysteinrest am C-Terminus, am N-Terminus, oder an einer Glykosylierungsstelle des Inhibitors enthält.

6. Verfahren nach Anspruch 5, wobei der TNF-Inhibitor die in Abbildung 19 dargestellte Aminosäuresequenz umfaßt und das nicht natürlich vorkommende Cystein am N-Terminus, C-Terminus oder den Aminosäurepositionen 1, 14 oder 105 ist.

45

7. Verfahren nach Anspruch 6, wobei der TNF-Inhibitor an ein polymeres Material mit hohem Molekulargewicht gebunden ist.

50

8. Verfahren nach Anspruch 7, wobei das polymere Material mit hohem Molekulargewicht Polyethylenglykol ist

9. Verfahren zum Herstellen einer pharmazeutischen Zubereitung, umfassend den TNF-Inhibitor, wobei der nach einem der Ansprüche 1 bis 8 hergestellte TNF-Inhibitor mit einem pharmazeutisch verträglichen Träger in einer per se bekannten Weise kombiniert wird.

55

10. Verfahren zum Herstellen einer Nukleinsäure, wobei die Nukleinsäure eine Sequenz aus der aus

- (i) der in Abbildung 13 dargestellten DNA-Sequenz
- (ii) der in Abbildung 20 dargestellten DNA-Sequenz
- (iii) der in Abbildung 21 dargestellten DNA-Sequenz
- (iv) der in Abbildung 37 dargestellten DNA-Sequenz
- (v) der in Abbildung 39 dargestellten DNA-Sequenz
- (vi) einem Fragment von (i), (ii), (iii), (iv) oder (v) das für ein Polypeptid mit TNF-inhibitorischer Aktivität kodiert
- (vii) einer Sequenz die im Vergleich zu den Sequenzen von (i), (ii), (iii), (iv), (v) oder (vi) degeneriert ist, die für ein Polypeptid mit TNF-inhibitorischer Aktivität kodiert
- (viii) einer Sequenz die zu (i), (ii), (iii), (iv), (v) (vi) oder (vii) komplementär ist
- (ix) einer Sequenz die mit (viii) hybridisiert und die für ein Polypeptid mit TNF-inhibitorischer Wirkung kodiert

bestehenden Gruppe ausgewählt ist.  
wird nach einer per se bekannten Weise synthetisiert.

- 11. Verfahren zum Herstellen eines rekombinanten Vektors, wobei eine Nukleinsäuresequenz wie nach Anspruch 10 erhältlich mit einem Vektor in einer per se bekannten Weise verbunden wird.
- 12. Verfahren zum Herstellen einer Wirtszelle, umfassend das Einführen einer DNA-Sequenz in eine gewünschte Wirtszelle um ein rekombinantes DNA-Molekül zu schaffen, das eine Nukleinsäuresequenz wie nach Anspruch 10 enthält.
- 13. Verfahren nach Anspruch 12, wobei die Wirtszelle eine prokaryotische Zelle ist.
- 14. Verfahren nach Anspruch 13, wobei die Wirtszelle ein *E. coli* ist.
- 15. Verfahren nach Anspruch 12, wobei die Wirtszelle eine eukaryotische Zelle ist.
- 16. Verfahren nach Anspruch 15, wobei die eukaryotische Zelle eine Säugerzelle ist.
- 17. Verfahren nach Anspruch 16, wobei die Säugerzelle eine Chinese hamster ovary-Zelle ist.

#### Patentansprüche für folgenden Vertragsstaat : GR

- 1. Tumor Nekrose-Faktor (TNF)-Inhibitor, umfassend die in Abbildung 19 dargestellte Aminosäuresequenz, wobei der Inhibitor in einer Wirtszelle hergestellt wird, die keine Glykosylierung durchführen kann oder die eine nicht-menschliche Zelle ist, welche Glykosylierung durchführen kann.
- 2. Tumor Nekrose-Faktor (TNF)-Inhibitor, der nicht glykosyliert ist und ein Molekulargewicht von circa 18 kDa hat.
- 3. TNF-Inhibitor nach Anspruch 2, wobei der TNF-Inhibitor durch rekombinante DNA-Verfahren hergestellt wird
- 4. TNF-Inhibitor, umfassend die Aminosäuresequenz wie dargestellt in Abbildung 38.  
wie dargestellt von Aminosäure 1 bis 235 (40 kDa-Inhibitor)  
oder von Aminosäure 1 bis 182 (40 kDa-Inhibitor Δ53) in Abbildung 38  
oder durch Aminosäuren 1 bis 184 wie in Abbildung 38 (40 kDa-Inhibitor Δ51) dargestellt.
- 5. TNF-Inhibitor nach Anspruch 4, wobei der TNF-Inhibitor in einer Wirtszelle hergestellt wird, die keine Glykosylierung durchführen kann oder die eine nicht-menschliche Zelle ist, welche Glykosylierung durchführen kann.
- 6. TNF-Inhibitor, umfassend die Aminosäuresequenz wie dargestellt in Abbildungen 19 oder 38 mit der Ausnahme daß die Aminosäure am N-Terminus, am C-Terminus oder an einer Glykosylierungsstelle durch einen Cysteinrest ersetzt ist.
- 7. TNF-Inhibitor nach Anspruch 6, wobei der TNF-Inhibitor eine die in Abbildung 19 dargestellte Aminosäuresequenz hat und das nicht natürlich vorkommende Cystein in Position Nr. 1, 14 oder 105 liegt

8. TNF-Inhibitor nach einem der Ansprüche 1 bis 3 und 6 wobei der TNF-Inhibitor an ein polymeres Material mit hohem Molekulargewicht gebunden ist

9. TNF-Inhibitor nach Anspruch 3 wobei das polymere Material mit hohem Molekulargewicht Polyethylenglykol ist

10. Verfahren zum Herstellen einer pharmazeutischen Zubereitung, umfassend das Kombinieren des TNF-Inhibitors gemäß einem der Ansprüche 1 bis 9 mit einem pharmazeutisch vertraglichen Träger.

11. Verfahren zum Herstellen eines TNF-Inhibitors, umfassend:

(a) Kultivieren einer Wirtszelle unter zur Expression des TNF-Inhibitors geeigneten Bedingungen, wobei die Wirtszelle eine Nukleinsäure enthält, die eine Sequenz umfaßt, die aus der aus:

(i) der in Abbildung 13 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(ii) der in Abbildung 20 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(iii) der in Abbildung 21 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(iv) der in Abbildung 37 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(v) der in Abbildung 39 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(vi) einer Sequenz, die in den kodierenden Bereichen von (i), (ii), (iii), (iv) und (v) oder Teilen davon degeneriert ist;

und

(vii) einer Sequenz, die an eine Nukleinsäure-Sequenz hybridisiert, die komplementär zu (i), (ii), (iii), (iv),

(v) oder (vi) ist:

bestehenden Gruppe ausgewählt ist:

(b) Ernten des Inhibitors.

12. Verfahren nach Anspruch 11, umfassend vor Schritt (a) weiterhin die Schritte:

I) Herstellen einer Nukleinsäure, die eine Sequenz umfaßt, die aus der aus:

(i) der in Abbildung 13 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(ii) der in Abbildung 20 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(iii) der in Abbildung 21 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(iv) der in Abbildung 37 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(v) der in Abbildung 39 dargestellten DNA-Sequenz oder einem kodierenden Teil davon:

(vi) einer Sequenz, die in den kodierenden Bereichen von (i), (ii), (iii), (iv) und (v) oder Teilen davon degeneriert ist;

und

(vii) einer Sequenz, die an eine Nukleinsäure-Sequenz hybridisiert, die komplementär zu (i), (ii), (iii), (iv),

(v) oder (vi) ist:

bestehenden Gruppe ausgewählt ist:

II) Klonieren der DNA-Sequenz in einen Vektor, der in eine vollständige Zelle übertragen und repliziert werden kann, wobei dieser Vektor funktionelle Elemente enthält, die zur Expression der DNA-Sequenz benötigt werden;

III) Übertragen des Vektors, der die synthetische DNA-Sequenz und funktionelle Elemente enthält, in einen Wirt, der die DNA, die für den TNF-Inhibitor kodiert, exprimieren kann

13. Verfahren nach Anspruch 11, weiter umfassend einen Schritt (c), in dem dem Inhibitor gestattet wird, eine aktive Tertiärstruktur anzunehmen, wodurch er TNF-inhibitorische Aktivität besitzt!

14. Verfahren nach Anspruch 11 wobei der TNF-Inhibitor eine Aminosäuresequenz umfaßt, die dargestellt ist in

(i) Abbildung 19

- (iii) Abbildung 38
  - (iii) Aminosäuren 1 bis 124, wie in Abbildung 38 (40kDa-Inhibitor ΔS1) dargestellt, oder
  - (iv) Aminosäuren 1 bis 122, wie in Abbildung 38 (40kDa-Inhibitor ΔS3) dargestellt
- 5 15. Verfahren nach Anspruch 11, wobei der TNF-Inhibitor einen nicht natürlich vorkommenden Cysteinrest am C-Terminus, am N-Terminus, oder an einer Glykosylierungsstelle des Inhibitors enthält.
16. Verfahren nach Anspruch 15, wobei der TNF-Inhibitor die in Abbildung 19 dargestellte Aminosäuresequenz umfaßt und das nicht natürlich vorkommende Cystein am N-Terminus, C-Terminus oder den Aminosäurepositionen 1, 14
- 10 oder 105 ist.
17. Nukleinsäure, umfassend eine Sequenz, die aus der aus:
- (i) der in Abbildung 13 dargestellten DNA-Sequenz
  - (ii) der in Abbildung 20 dargestellten DNA-Sequenz
  - (iii) der in Abbildung 21 dargestellten DNA-Sequenz
  - (iv) der in Abbildung 37 dargestellten DNA-Sequenz
  - (v) der in Abbildung 39 dargestellten DNA-Sequenz
  - (vi) einem Fragment von (i), (ii), (iii), (iv) oder (v), das für ein Polypeptid mit TNF-inhibitorischer Aktivität kodiert
  - (vii) einer Sequenz, die im Vergleich zu den Sequenzen von (i), (ii), (iii), (iv), (v) oder (vi) degeneriert ist, die für ein Polypeptid mit TNF-inhibitorischer Aktivität kodiert
  - (viii) einer Sequenz, die zu (i), (ii), (iii), (iv), (v) (vi) oder (vii) komplementär ist
  - (ix) einer Sequenz, die mit (viii) hybridisiert und die für ein Polypeptid mit TNF-inhibitorischer Wirkung kodiert
- 15 bestehenden Gruppe ausgewählt ist.
18. TNF-Inhibitor nach einem der Ansprüche 1 bis 9 zur Verwendung beim Behandeln einer TNF-vermittelten Krankheit.
19. TNF-Inhibitor nach einem der Ansprüche 1 bis 9 zur Verwendung in Kombination mit einem IL-1-Inhibitor beim Behandeln einer TNF-vermittelten Krankheit.
20. Verwendung des TNF-Inhibitors nach einem der Ansprüche 1 bis 9 zum Herstellen eines Medikamentes zum Behandeln oder Vorbeugen von TNF-vermittelten Krankheiten.
21. Verwendung nach Anspruch 20, wobei das Medikament weiterhin einen IL-1-Inhibitor umfaßt.
22. Vektor, umfassend eine Nukleinsäure-Sequenz wie in Anspruch 17 definiert.
23. Wirtszelle, enthaltend ein rekombinantes DNA-Molekül, umfassend eine Nukleinsäure-Sequenz wie in Anspruch 17 definiert.
24. Wirtszelle nach Anspruch 23, die eine prokaryotische Zelle ist.
25. Wirtszelle nach Anspruch 24, die ein *E. coli* ist.
26. Wirtszelle nach Anspruch 23, die eine eukaryotische Zelle ist.
27. Wirtszelle nach Anspruch 26, wobei die eukaryotische Zelle eine Säugerzelle ist.
28. Wirtszelle nach Anspruch 27, wobei die Säugerzelle eine Chinese hamster ovary-Zelle ist.

## Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, GB, IT, LI, LU, NL, SE

1. Inhibiteur du facteur de nécrose tumorale (TNF) comprenant la séquence d'acides aminés présentée sur la Figure 19 ou ledit inhibiteur est produit dans une cellule hôte qui n'est pas capable de glycosylation ou qui est une cellule non-humaine capable de glycosylation
2. Inhibiteur du facteur de nécrose tumorale (TNF), qui est non-glycosylé et a une masse moléculaire d'environ 15 kDa.
3. Inhibiteur du TNF selon la revendication 2, dans lequel ledit inhibiteur du TNF est produit par les méthodes de recombinaison de l'ADN.
4. Inhibiteur du TNF comprenant la séquence d'acides aminés représentée par les résidus 1 à 235 de la Figure 38 (inhibiteur de 40 kDa), par les résidus 1 à 182 de la Figure 38 (inhibiteur Δ53 de 40 kDa), ou par les résidus 1 à 184 de la Figure 38 (inhibiteur Δ51 de 40 kDa).
5. Inhibiteur du TNF selon la revendication 4, dans lequel ledit inhibiteur du TNF est produit dans une cellule hôte qui n'est pas capable de glycosylation, ou qui est une cellule non-humaine capable de glycosylation
6. Inhibiteur du TNF comprenant la séquence d'acides aminés représentée sur les Figures 19 ou 38, sauf que l'acide aminé sur le site N-terminal, sur le site C-terminal ou sur un site de glycosylation, est remplacé par un résidu de cystéine.
7. Inhibiteur du TNF selon la revendication 6, dans lequel ledit inhibiteur du TNF est la séquence d'acides aminés représentée sur la Figure 19, et ladite cystéine non-naturelle se trouve sur les numéros de résidu 1, 14 ou 105
8. Inhibiteur du TNF selon l'une quelconque des revendications 1 à 3 et 6, dans lequel ledit inhibiteur du TNF est lié à un matériau polymère à grande masse moléculaire.
9. Inhibiteur du TNF selon la revendication 8, dans lequel ledit matériau polymère à grande masse moléculaire est le polyéthyléneglycol.
10. Préparation pharmaceutique comprenant l'inhibiteur du TNF selon l'une quelconque des revendications 1 à 9 dans un excipient acceptable d'un point de vue pharmaceutique.
11. Procédé pour produire un inhibiteur du TNF, qui comprend :
  - (a) à cultiver dans des conditions convenant à l'expression dudit inhibiteur du TNF une cellule-hôte contenant un acide nucléique comprenant une séquence choisie parmi l'ensemble des séquences suivantes
    - (i) la séquence d'ADN représentée sur la Figure 13 ou une portion codante de cette séquence
    - (ii) la séquence d'ADN représentée sur la Figure 20 ou une portion codante de cette séquence
    - (iii) la séquence d'ADN représentée sur la Figure 21 ou une portion codante de cette séquence
    - (iv) la séquence d'ADN représentée sur la Figure 37 ou une portion codante de cette séquence
    - (v) la séquence d'ADN représentée sur la Figure 39 ou une portion codante de cette séquence
    - (vi) une séquence qui est dégénérée dans les régions codantes ou leurs portions de (i), (ii), (iii), (iv) et (v)
    - (vii) une séquence qui s'hybride à une séquence d'acides nucléiques qui est complémentaire de (i), (ii), (iii), (iv), (v) ou (vi)
  - (b) à récolter ledit inhibiteur
12. Procédé selon la revendication 11, qui comprend en outre, avant l'étape (a), les étapes consistant
  - i) à préparer un acide nucléique comprenant une séquence choisie parmi l'ensemble de séquences suivant

- (i) la séquence d'ADN représentée sur la Figure 13 ou une portion codante de cette séquence  
(ii) la séquence d'ADN représentée sur la Figure 20 ou une portion codante de cette séquence  
(iii) la séquence d'ADN représentée sur la Figure 21 ou une portion codante de cette séquence  
(iv) la séquence d'ADN représentée sur la Figure 37 ou une portion codante de cette séquence  
(v) une séquence qui est dégénérée dans les régions codantes ou leurs portions de (i), (ii), (iii), (iv) et (v)  
(vi) une séquence qui s'hybride à une séquence d'acides nucléiques qui est complémentaire de (i), (ii), (iii), (iv), (v) ou (vi).
- II) a cloner la séquence d'ADN dans un vecteur pouvant être transféré et répliqué dans une cellule entière  
ledit vecteur contenant des éléments opérationnels nécessaires à l'expression de la séquence d'ADN  
III) à transférer le vecteur contenant la séquence d'ADN de synthèse et les éléments opérationnels dans un hôte capable d'exprimer l'ADN codant l'inhibiteur du TNF.
13. Procédé selon la revendication 11, qui comprend en outre l'étape consistant  
(c) à permettre à l'inhibiteur de prendre une structure tertiaire active, grâce à laquelle il possède une activité inhibitrice du TNF.
14. Procédé selon la revendication 11, dans lequel ledit inhibiteur du TNF comprend la séquence d'acides aminés représentée  
(i) sur la Figure 19.  
(ii) sur la Figure 38.  
(iii) par les résidus 1 à 184 ressortant de la  
Figure 38 (inhibiteur  $\Delta 51$  de 40 kDa), ou (iv) par les résidus 1 à 182 ressortant de la Figure 38 (inhibiteur  $\Delta 53$  de 40 kDa).
15. Procédé selon la revendication 11, dans lequel ledit inhibiteur du TNF a un résidu de cystéine non-naturel sur le site C-terminal, le site N-terminal ou un site de glycosylation dudit inhibiteur.
16. Procédé selon la revendication 15, dans lequel ledit inhibiteur du TNF comprend la séquence d'acides aminés représentée sur la Figure 19, et ladite cystéine non-naturelle se trouve sur le site N-terminal, sur le site C-terminal ou sur les numéros de résidu 1, 14 ou 105.
17. Acide nucléique comprenant une séquence choisie parmi les séquences suivantes :  
(i) la séquence d'ADN représentée sur la Figure 13.  
(ii) la séquence d'ADN représentée sur la Figure 20.  
(iii) la séquence d'ADN représentée sur la Figure 21.  
(iv) la séquence d'ADN représentée sur la Figure 37.  
(v) la séquence d'ADN représentée sur la Figure 39.  
(vi) un fragment de (i), (ii), (iii), (iv) ou (v) codant un polypeptide ayant une activité inhibitrice du TNF.  
(vii) une séquence qui est un produit de dégénérescence de (i), (ii), (iii), (iv), (v) ou (vi), codant un polypeptide ayant une activité inhibitrice du TNF.  
(viii) une séquence complémentaire de (i), (ii), (iii), (iv), (v), (vi) ou (vii).  
(ix) une séquence qui s'hybride à (viii) et qui code un polypeptide ayant une activité inhibitrice du TNF.
18. Inhibiteur du TNF selon l'une quelconque des revendications 1 à 9, pour utilisation dans le traitement d'une maladie à médiation par le TNF.
19. Inhibiteur du TNF selon l'une quelconque des revendications 1 à 9, pour utilisation dans le traitement d'une maladie à médiation par le TNF en combinaison avec un inhibiteur de l'IL-1.
20. Utilisation de l'inhibiteur du TNF selon l'une quelconque des revendications 1 à 9 pour préparer un médicament destiné à traiter ou prévenir les maladies à médiation par le TNF.
21. Utilisation selon la revendication 20, dans laquelle ledit médicament comprend en outre un inhibiteur de l'IL-1.

22. Vecteur comprenant une séquence d'acides nucléiques selon la revendication 17
23. Cellule hôte contenant une molécule d'ADN recombinant, comprenant une séquence d'acide nucléique selon la revendication 17
24. Cellule hôte selon la revendication 23, qui est une cellule procaryote.
25. Cellule hôte selon la revendication 24, qui est un *E. coli*.
26. Cellule hôte selon la revendication 23, qui est une cellule eucaryote.
27. Cellule hôte selon la revendication 26, dans laquelle ladite cellule eucaryote est une cellule de mammifère
28. Cellule hôte selon la revendication 27, dans laquelle ladite cellule de mammifère est une cellule de l'ovaire du hamster chinois.

#### Revendications pour l'Etat contractant suivant : ES

1. Procédé pour produire un inhibiteur du TNF qui comprend :
- (a) à cultiver dans des conditions convenant à l'expression dudit inhibiteur du TNF une cellule-hôte contenant un acide nucléique comprenant une séquence choisie parmi l'ensemble des séquences suivantes :
- (i) la séquence d'ADN représentée sur la Figure 13 ou une portion codante de cette séquence ;
  - (ii) la séquence d'ADN représentée sur la Figure 20 ou une portion codante de cette séquence ;
  - (iii) la séquence d'ADN représentée sur la Figure 21 ou une portion codante de cette séquence ;
  - (iv) la séquence d'ADN représentée sur la Figure 37 ou une portion codante de cette séquence ;
  - (v) la séquence d'ADN représentée sur la Figure 39 ou une portion codante de cette séquence ;
  - (vi) une séquence qui est dégénérée dans les régions codantes ou leurs portions de (i), (ii), (iii), (iv) et (v) ;
  - (vii) une séquence qui s'hybride à une séquence d'acides nucléiques qui est complémentaire de (i), (ii), (iii), (iv), (v) ou (vi) ;
- (b) à récolter ledit inhibiteur.
2. Procédé selon la revendication 1, qui comprend en outre, avant l'étape (a), les étapes consistant :
- I) à préparer un acide nucléique comprenant une séquence choisie parmi l'ensemble de séquences suivant :
- (i) la séquence d'ADN représentée sur la Figure 13 ou une portion codante de cette séquence
  - (ii) la séquence d'ADN représentée sur la Figure 20 ou une portion codante de cette séquence
  - (iii) la séquence d'ADN représentée sur la Figure 21 ou une portion codante de cette séquence
  - (iv) la séquence d'ADN représentée sur la Figure 37 ou une portion codante de cette séquence
  - (v) la séquence d'ADN représentée sur la Figure 39 ou une portion codante de cette séquence
  - (vi) une séquence qui est dégénérée dans les régions codantes ou leurs portions de (i), (ii), (iii), (iv) et (v)
  - (vii) une séquence qui s'hybride à une séquence d'acides nucléiques qui est complémentaire de (i), (ii), (iii), (iv), (v) ou (vi)
- II) à cloner la séquence d'ADN dans un vecteur pouvant être transféré et répliqué dans une cellule entière ledit vecteur contenant des éléments opérationnels nécessaires à l'expression de la séquence d'ADN
- III) à transférer le vecteur contenant la séquence d'ADN de synthèse et les éléments opérationnels dans un hôte capable d'exprimer l'ADN codant l'inhibiteur du TNF.
3. Procédé selon la revendication 1, qui comprend en outre l'étape consistant
- (c) à permettre à l'inhibiteur de prendre une structure tertiaire active grâce à laquelle il possède une activité inhibitrice du TNF
4. Procédé selon la revendication 1, dans lequel ledit inhibiteur du TNF comprend la séquence d'acides aminés



représentée

(i) sur la Figure 19

(iii) sur la Figure 38

5 (iii) par les résidus 1 à 134 ressortant de la Figure 38 (inhibiteur Δ51 de 40 kDa), ou

(iv) par les résidus 1 à 132 ressortant de la Figure 38 (inhibiteur Δ53 de 40 kDa).

10 5. Procédé selon la revendication 1, dans lequel ledit inhibiteur du TNF a un résidu de cystéine non-naturel sur le site C-terminal, le site N-terminal ou un site de glycosylation dudit inhibiteur

6. Procédé selon la revendication 5, dans lequel ledit inhibiteur du TNF comprend la séquence d'acides aminés représentée sur la Figure 19, et ladite cystéine non-naturelle se trouve sur le site N-terminal, sur le site C-terminal ou sur les numéros de résidu 1, 14 ou 105.

15 7. Procédé selon la revendication 6, dans lequel ledit inhibiteur du TNF est lié à un matériau polymère à grande masse moléculaire.

8. Procédé selon la revendication 7, dans lequel ledit matériau polymère à grande masse moléculaire est le polyéthylène glycol.

20

9. Procédé pour préparer une composition pharmaceutique comprenant l'inhibiteur du TNF, dans lequel l'inhibiteur du TNF produit selon l'une quelconque des revendications 1 à 8 est combiné d'une manière connue en soi à un excipient acceptable d'un point de vue pharmaceutique.

25 10. Procédé pour préparer un acide nucléique, dans lequel on synthétise d'une manière connue en soi un acide nucléique comprenant une séquence choisie parmi les séquences suivantes :

(i) la séquence d'ADN représentée sur la Figure 13.

(ii) la séquence d'ADN représentée sur la Figure 20.

30

(iii) la séquence d'ADN représentée sur la Figure 21.

(iv) la séquence d'ADN représentée sur la Figure 37.

(v) la séquence d'ADN représentée sur la Figure 39.

(vi) un fragment de (i), (ii), (iii), (iv) ou (v) codant un polypeptide ayant une activité inhibitrice du TNF.

35

(vii) une séquence qui est un produit de dégénérescence de (i), (ii), (iii), (iv), (v) ou (vi), codant un polypeptide ayant une activité inhibitrice du TNF.

(viii) une séquence complémentaire de (i), (ii), (iii), (iv), (v), (vi) ou (vii).

(ix) une séquence qui s'hybride à (viii) et qui code un polypeptide ayant une activité inhibitrice du TNF.

40

11. Procédé pour préparer un vecteur recombinant, dans lequel on réunit à un ADN de vecteur d'une manière connue en soi une séquence d'acides nucléiques telle que pouvant être obtenue selon la revendication 10.

45

12. Procédé pour préparer une cellule hôte, qui comprend à introduire dans une cellule hôte voulue une séquence d'ADN pour générer une molécule d'ADN recombinant contenant une séquence d'acides nucléiques telle que pouvant être obtenue selon la revendication 10.

50

13. Procédé selon la revendication 12, dans lequel la cellule hôte est une cellule procaryote.

14. Procédé selon la revendication 13, dans lequel la cellule hôte est un *E. coli*.

55

15. Procédé selon la revendication 12, dans lequel la cellule hôte est une cellule eucaryote.

16. Procédé selon la revendication 15, dans lequel ladite cellule eucaryote est une cellule de mammifère.

17. Procédé selon la revendication 16, dans lequel ladite cellule de mammifère est une cellule de l'ovaire du hamster chinois.

## Revendications pour l'Etat contractant suivant : GR

1. Inhibiteur du facteur de nécrose tumorale (TNF) comprenant la séquence d'acides aminés présentée sur la Figure 19 ou ledit inhibiteur est produit dans une cellule hôte qui n'est pas capable de glycosylation ou qui est une cellule non-humaine capable de glycosylation.
2. Inhibiteur du facteur de nécrose tumorale (TNF) qui est non-glycosylé et a une masse moléculaire d'environ 13 kDa.
3. Inhibiteur du TNF selon la revendication 2, dans lequel ledit inhibiteur du TNF est produit par les méthodes de recombinaison de l'ADN.
4. Inhibiteur du TNF comprenant la séquence d'acides aminés représentée par les résidus 1 à 235 de la Figure 38 (inhibiteur de 40 kDa), ou par les résidus 1 à 182 de la Figure 38 (inhibiteur  $\Delta$ 53 de 40 kDa), ou par les résidus 1 à 184 de la Figure 38 (inhibiteur  $\Delta$ 51 de 40 kDa).
5. Inhibiteur du TNF selon la revendication 4, dans lequel ledit inhibiteur du TNF est produit dans une cellule hôte qui n'est pas capable de glycosylation, ou qui est une cellule non-humaine capable de glycosylation.
6. Inhibiteur du TNF comprenant la séquence d'acides aminés ressortant des Figures 19 ou 38, sauf que l'acide aminé sur le site N-terminal, sur le site C-terminal ou sur un site de glycosylation, est remplacé par un résidu de cystéine.
7. Inhibiteur du TNF selon la revendication 6, dans lequel ledit inhibiteur du TNF a la séquence d'acides aminés représentée sur la Figure 19, et ladite cystéine non-naturelle se trouve sur les numéros de résidu 1, 14 ou 105.
8. Inhibiteur du TNF selon l'une quelconque des revendications 1 à 3 et 6, dans lequel ledit inhibiteur du TNF est lié à un matériau polymère à grande masse moléculaire.
9. Inhibiteur du TNF selon la revendication 8, dans lequel ledit matériau polymère à grande masse moléculaire est le polyéthylèneglycol.
10. Procédé pour préparer une préparation pharmaceutique, qui comprend à combiner l'inhibiteur du TNF selon l'une quelconque des revendications 1 à 9 et un excipient acceptable d'un point de vue pharmaceutique.
11. Procédé pour produire un inhibiteur du TNF qui comprend :
  - (a) à cultiver dans des conditions convenant à l'expression dudit inhibiteur du TNF une cellule-hôte contenant un acide nucléique comprenant une séquence choisie parmi l'ensemble des séquences suivantes
    - (i) la séquence d'ADN représentée sur la Figure 13 ou une portion codante de cette séquence
    - (ii) la séquence d'ADN représentée sur la Figure 20 ou une portion codante de cette séquence
    - (iii) la séquence d'ADN représentée sur la Figure 21 ou une portion codante de cette séquence
    - (iv) la séquence d'ADN représentée sur la Figure 37 ou une portion codante de cette séquence
    - (v) la séquence d'ADN représentée sur la Figure 39 ou une portion codante de cette séquence
    - (vi) une séquence qui est dégénérée dans les régions codantes ou leurs portions de (i), (ii), (iii), (iv) et (v),
    - (vii) une séquence qui s'hybride à une séquence d'acides nucléiques qui est complémentaire de (i), (ii), (iii), (iv), (v) ou (vi) ;
  - (b) à récolter ledit inhibiteur.
12. Procédé selon la revendication 11, qui comprend en outre, avant l'étape (a), les étapes consistant
  - I) à préparer un acide nucléique comprenant une séquence choisie parmi l'ensemble de séquences suivant
    - (i) la séquence d'ADN représentée sur la Figure 13 ou une portion codante de cette séquence
    - (ii) la séquence d'ADN représentée sur la Figure 20 ou une portion codante de cette séquence
    - (iii) la séquence d'ADN représentée sur la Figure 21 ou une portion codante de cette séquence

- (iv) la séquence d'ADN représentée sur la Figure 37 ou une portion codante de cette séquence  
 (v) la séquence d'ADN représentée sur la Figure 39 ou une portion codante de cette séquence  
 (vi) une séquence qui est dégénérée dans les régions codantes ou leurs portions de (i), (ii), (iii), (iv) et (v);  
 (vii) une séquence qui s'hybride à une séquence d'acides nucléiques qui est complémentaire de (i), (ii),  
 5 (iii), (iv), (v) ou (vi).
- (i) à cloner la séquence d'ADN dans un vecteur pouvant être transféré et répliqué dans une cellule entière  
 ledit vecteur contenant des éléments opérationnels nécessaires à l'expression de la séquence d'ADN  
 (iii) à transférer le vecteur contenant la séquence d'ADN de synthèse et les éléments opérationnels dans un  
 10 hôte capable d'exprimer l'ADN codant l'inhibiteur du TNF.
13. Procédé selon la revendication 11, qui comprend en outre l'étape consistant  
 (c) à permettre à l'inhibiteur de prendre une structure tertiaire active, grâce à laquelle il possède une activité  
 15 inhibitrice du TNF.
14. Procédé selon la revendication 11, dans lequel ledit inhibiteur du TNF comprend la séquence d'acides aminés  
 représentée  
 20 (i) sur la Figure 19,  
 (ii) sur la Figure 38,  
 (iii) par les résidus 1 à 184 ressortant de la Figure 38 (inhibiteur Δ51 de 40 kDa) ou  
 (iv) par les résidus 1 à 182 ressortant de la Figure 38 (inhibiteur Δ53 de 40 kDa).
15. Procédé selon la revendication 11, dans lequel ledit inhibiteur du TNF a un résidu de cystéine non-naturel sur le  
 25 site C-terminal, le site N-terminal ou un site de glycosylation dudit inhibiteur.
16. Procédé selon la revendication 15, dans lequel ledit inhibiteur du TNF comprend la séquence d'acides aminés  
 représentée sur la Figure 19, et ladite cystéine non-naturelle se trouve sur le site N-terminal, sur le site C-terminal  
 30 ou sur les numéros de résidu 1, 14 ou 105.
17. Acide nucléique comprenant une séquence choisie parmi les séquences suivantes  
 35 (i) la séquence d'ADN représentée sur la Figure 13,  
 (ii) la séquence d'ADN représentée sur la Figure 20,  
 (iii) la séquence d'ADN représentée sur la Figure 21,  
 (iv) la séquence d'ADN représentée sur la Figure 37,  
 (v) la séquence d'ADN représentée sur la Figure 39,  
 (vi) un fragment de (i), (ii), (iii), (iv) ou (v) codant un polypeptide ayant une activité inhibitrice du TNF,  
 (vii) une séquence qui est un produit de dégenérescence de (i), (ii), (iii), (iv), (v) ou (vi), codant un polypeptide  
 40 ayant une activité inhibitrice du TNF,  
 (viii) une séquence complémentaire de (i), (ii), (iii), (iv), (v), (vi) ou (vii),  
 (ix) une séquence qui s'hybride à (viii) et qui code un polypeptide ayant une activité inhibitrice du TNF.
18. Inhibiteur du TNF selon l'une quelconque des revendications 1 à 9, pour utilisation dans le traitement d'une maladie  
 45 à médiation par le TNF.
19. Inhibiteur du TNF selon l'une quelconque des revendications 1 à 9, pour utilisation dans le traitement d'une maladie  
 à médiation par le TNF en combinaison avec un inhibiteur de l'IL-1.
20. Utilisation de l'inhibiteur du TNF selon l'une quelconque des revendications 1 à 9 pour préparer un médicament  
 50 destiné à traiter ou prévenir les maladies à médiation par le TNF.
21. Utilisation selon la revendication 20, dans laquelle ledit médicament comprend en outre un inhibiteur de l'IL-1.
22. Vecteur comprenant une séquence d'acides nucléiques selon la revendication 17  
 55
23. Cellule hôte contenant une molécule d'ADN recombinant, comprenant une séquence d'acide nucléique selon la  
 revendication 17.

24. Cellule hôte selon la revendication 23, qui est une cellule procaryote

25. Cellule hôte selon la revendication 24 qui est un *E. coli*

5 26. Cellule hôte selon la revendication 23 qui est une cellule eucaryote

27. Cellule hôte selon la revendication 26 dans laquelle ladite cellule eucaryote est une cellule de mammifère

10 28. Cellule hôte selon la revendication 27 dans laquelle ladite cellule de mammifère est une cellule de l'ovaire du hamster chinois

15

20

25

30

35

40

45

50

55

FIG. 1

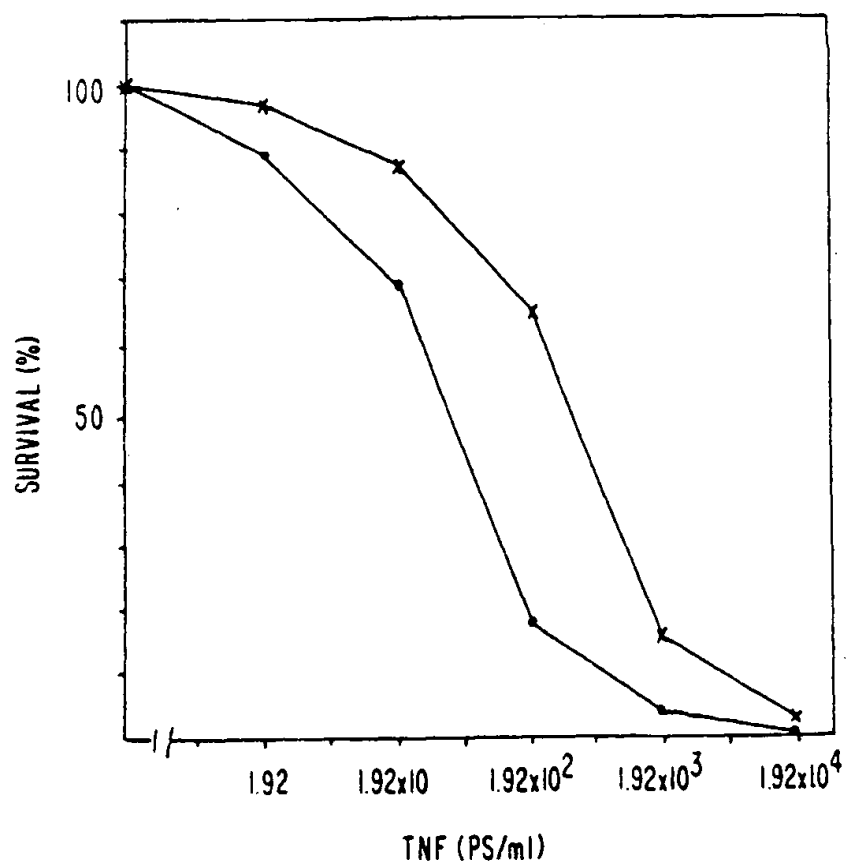


FIG. 2



FIG. 3



FIG.4

a b c d



FIG.5

a b

KDa

43

29

18

14



FIG. 6A

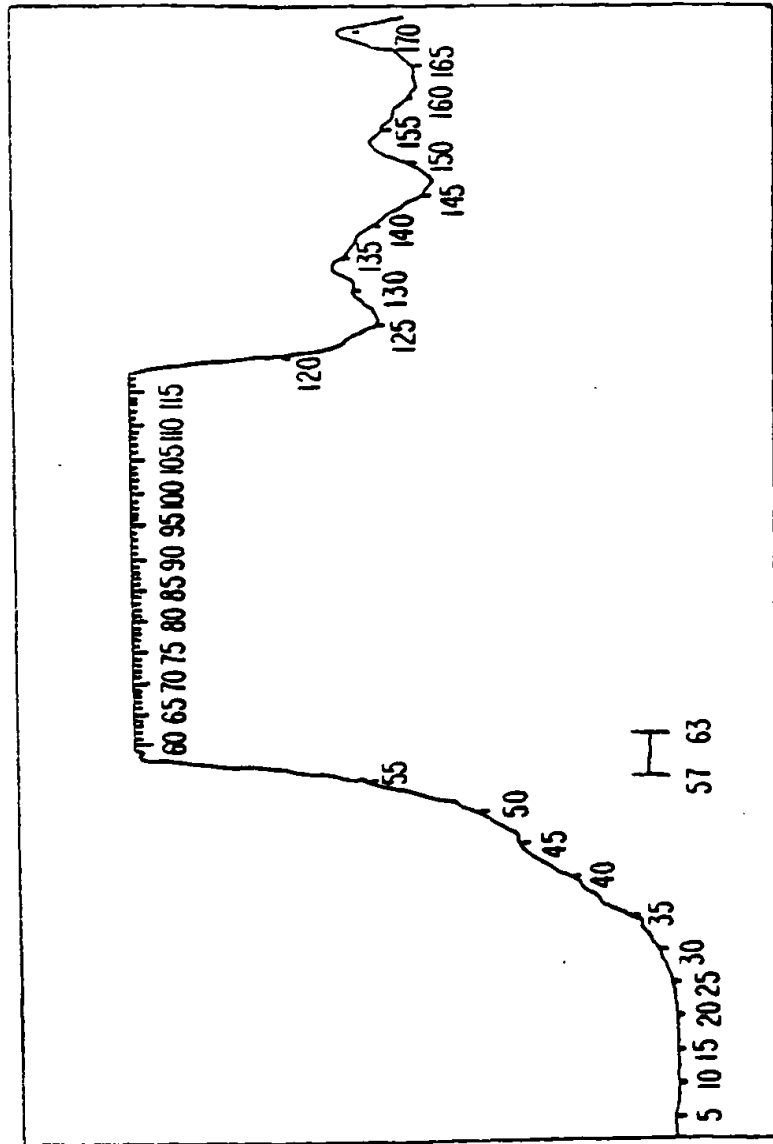




FIG. 6B

45 47 49 51 53 55 57 59 61 63 65

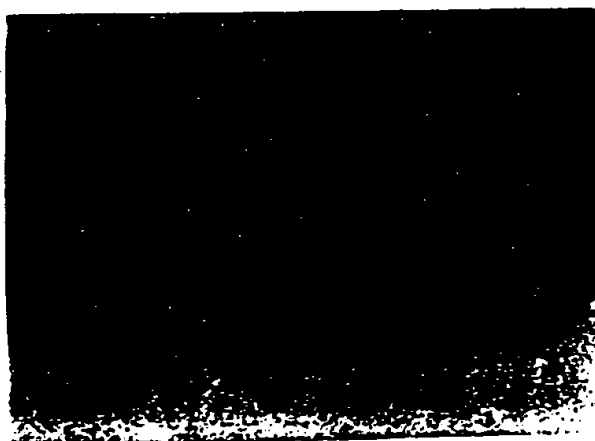


FIG. 7

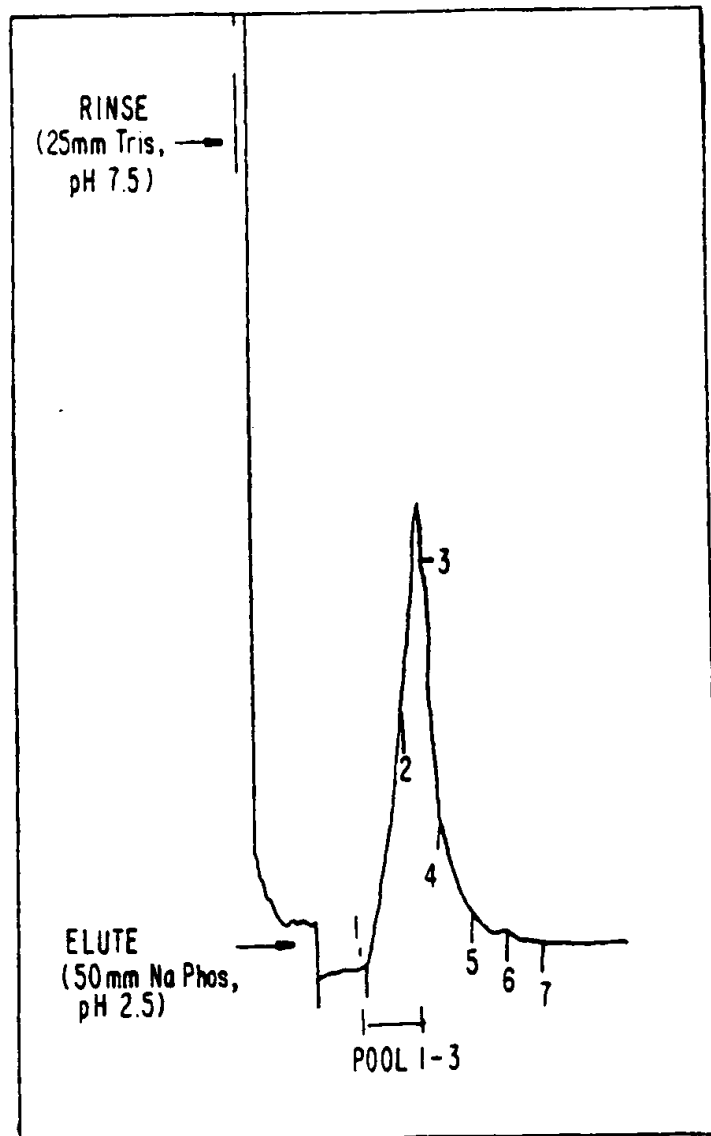


FIG. 8A

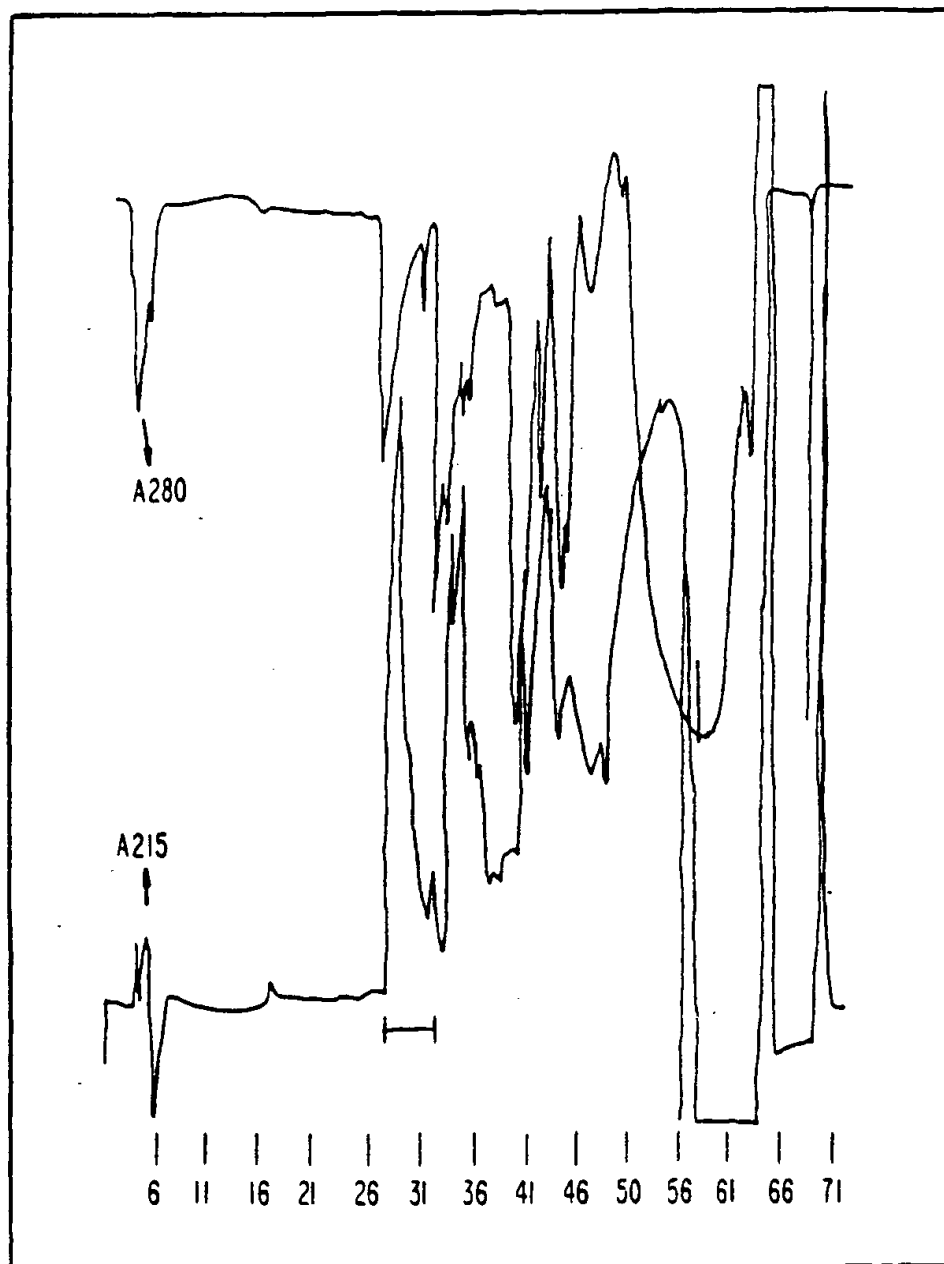


FIG. 8B

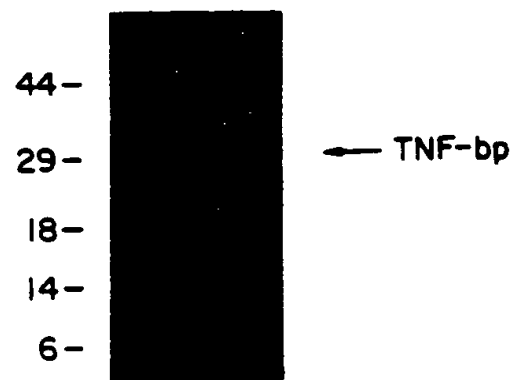


FIG. 8C

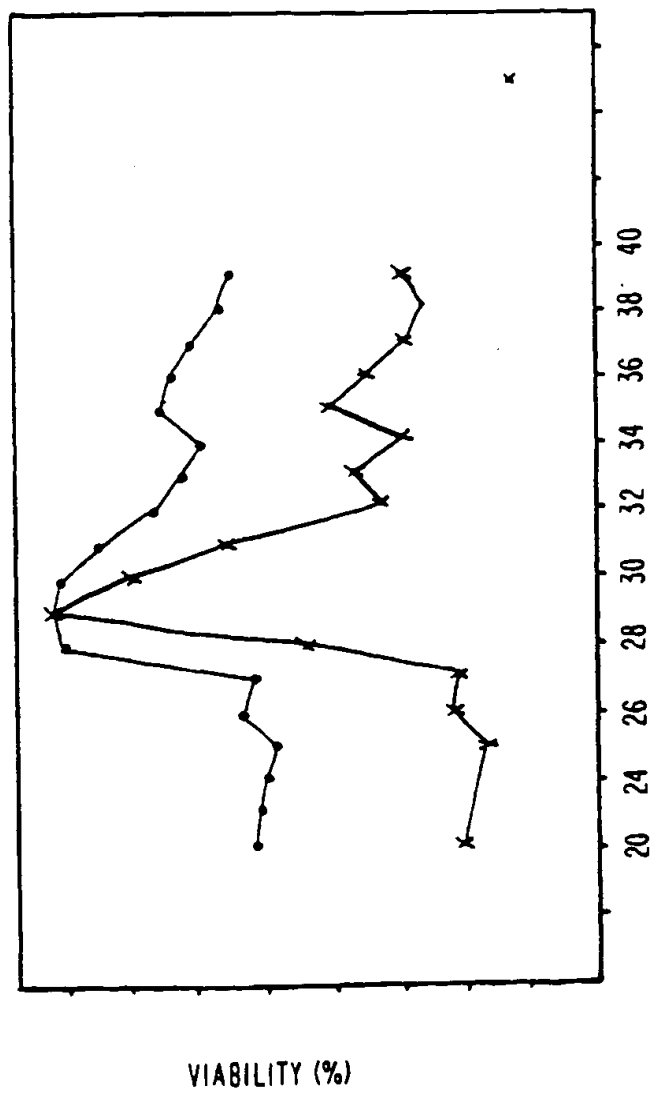


FIG. 9A

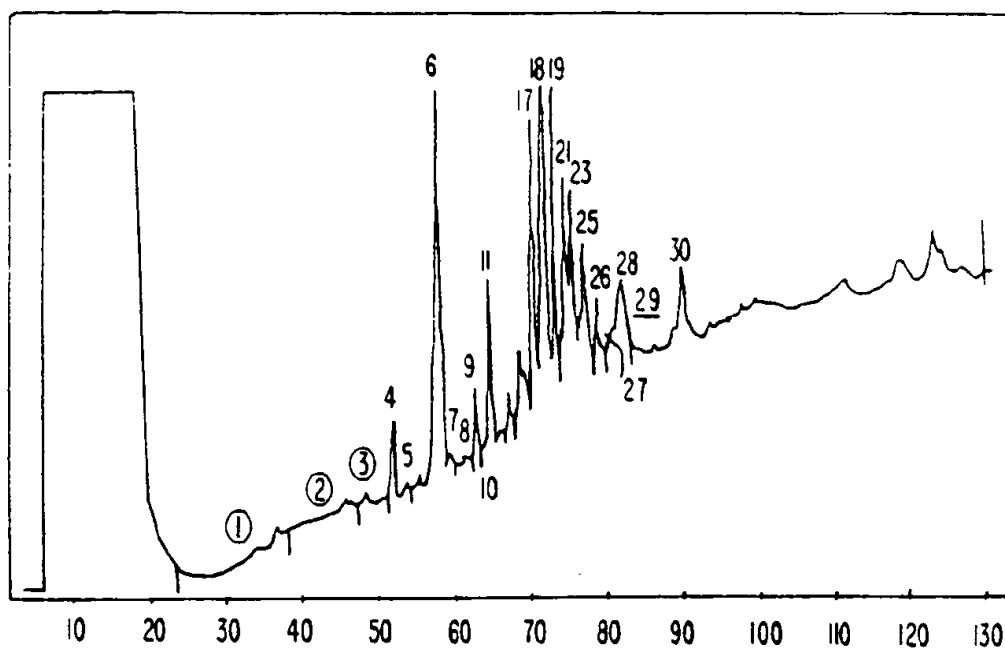


FIG. 9B-1

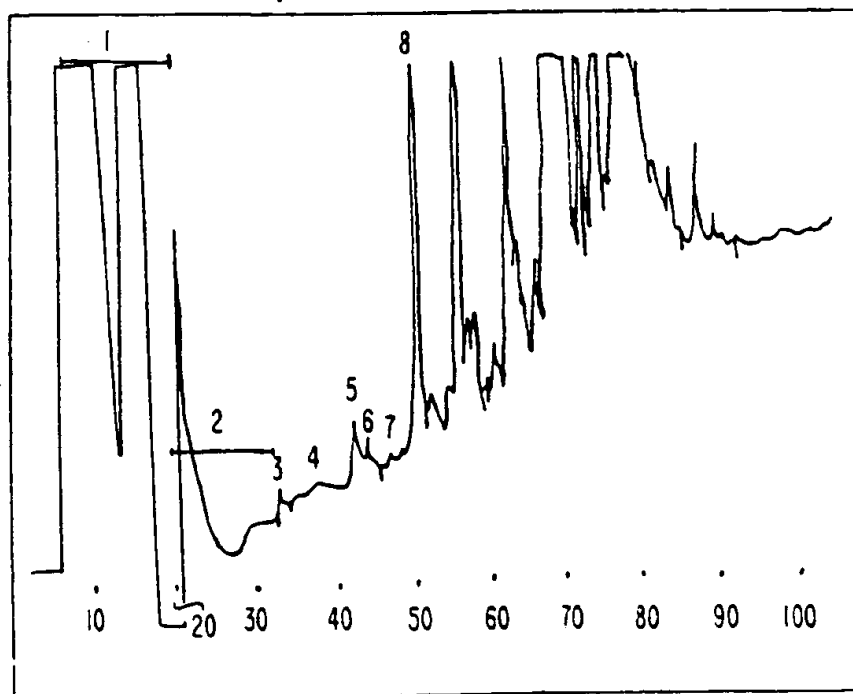


FIG. 9B-2

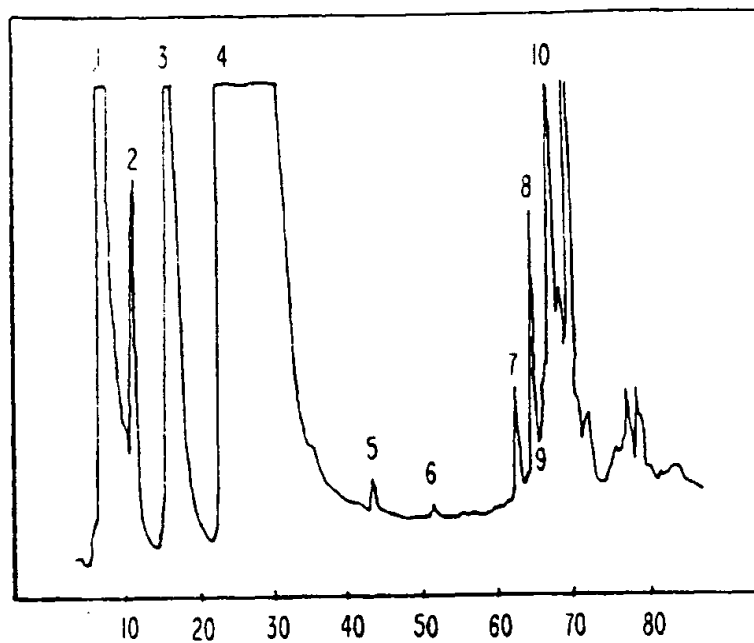


FIG. 9B-3

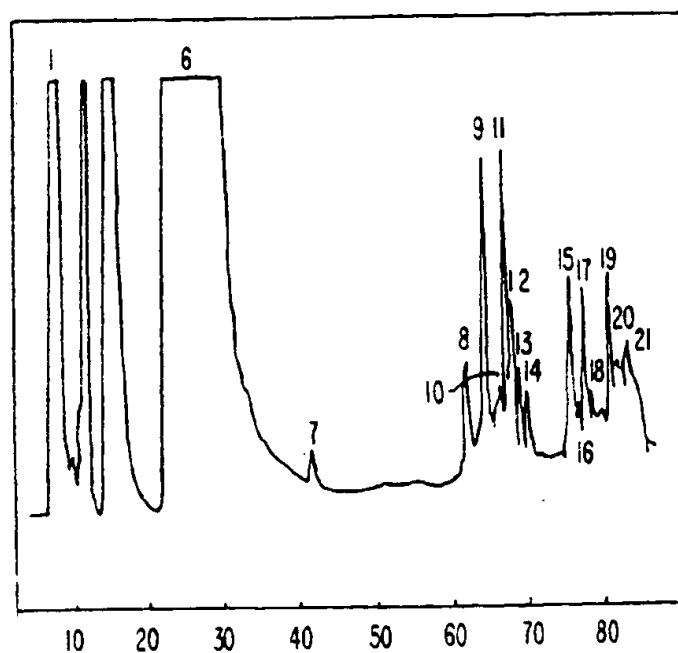


FIG. 10A

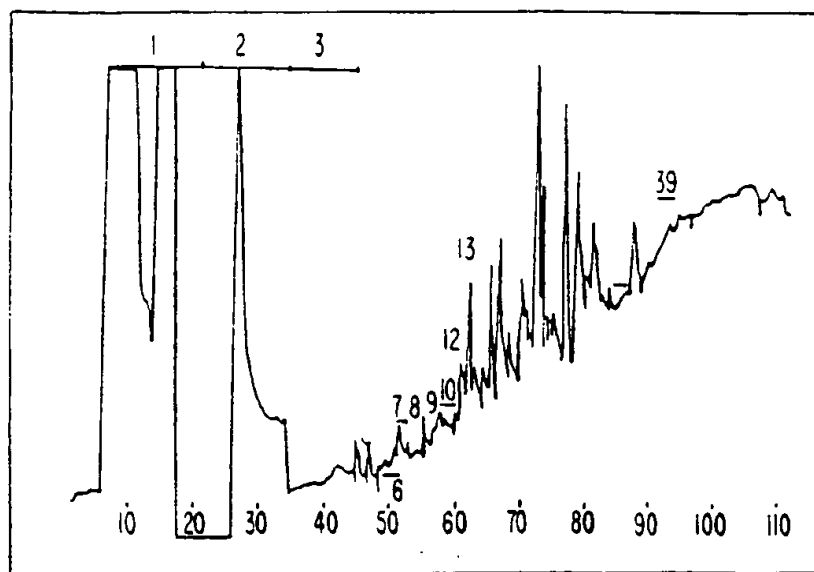


FIG. 10B

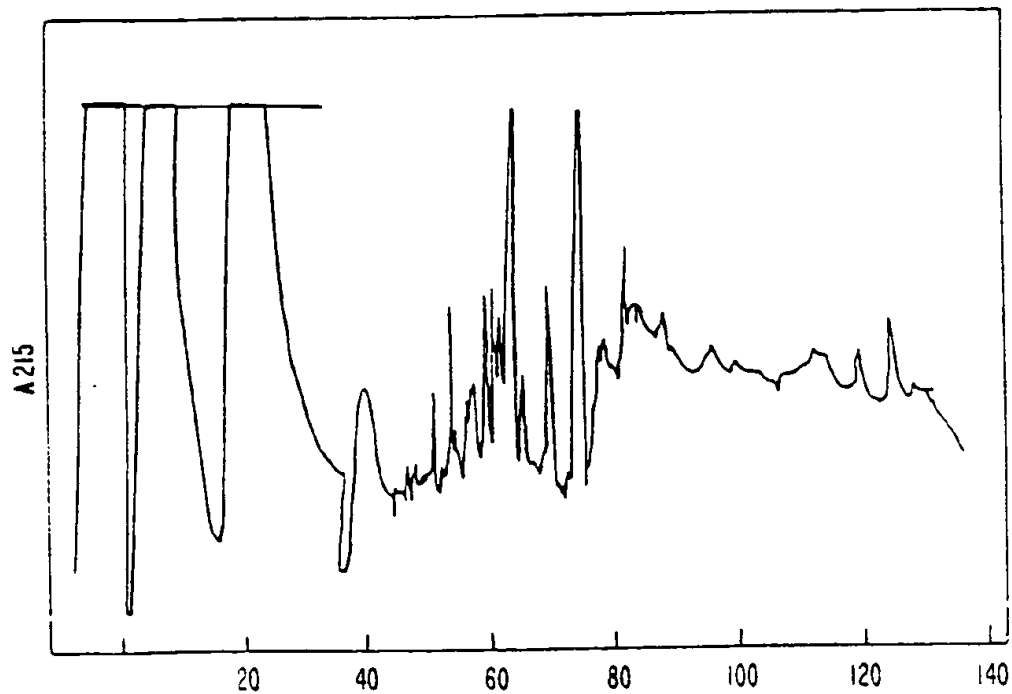




FIG.11A

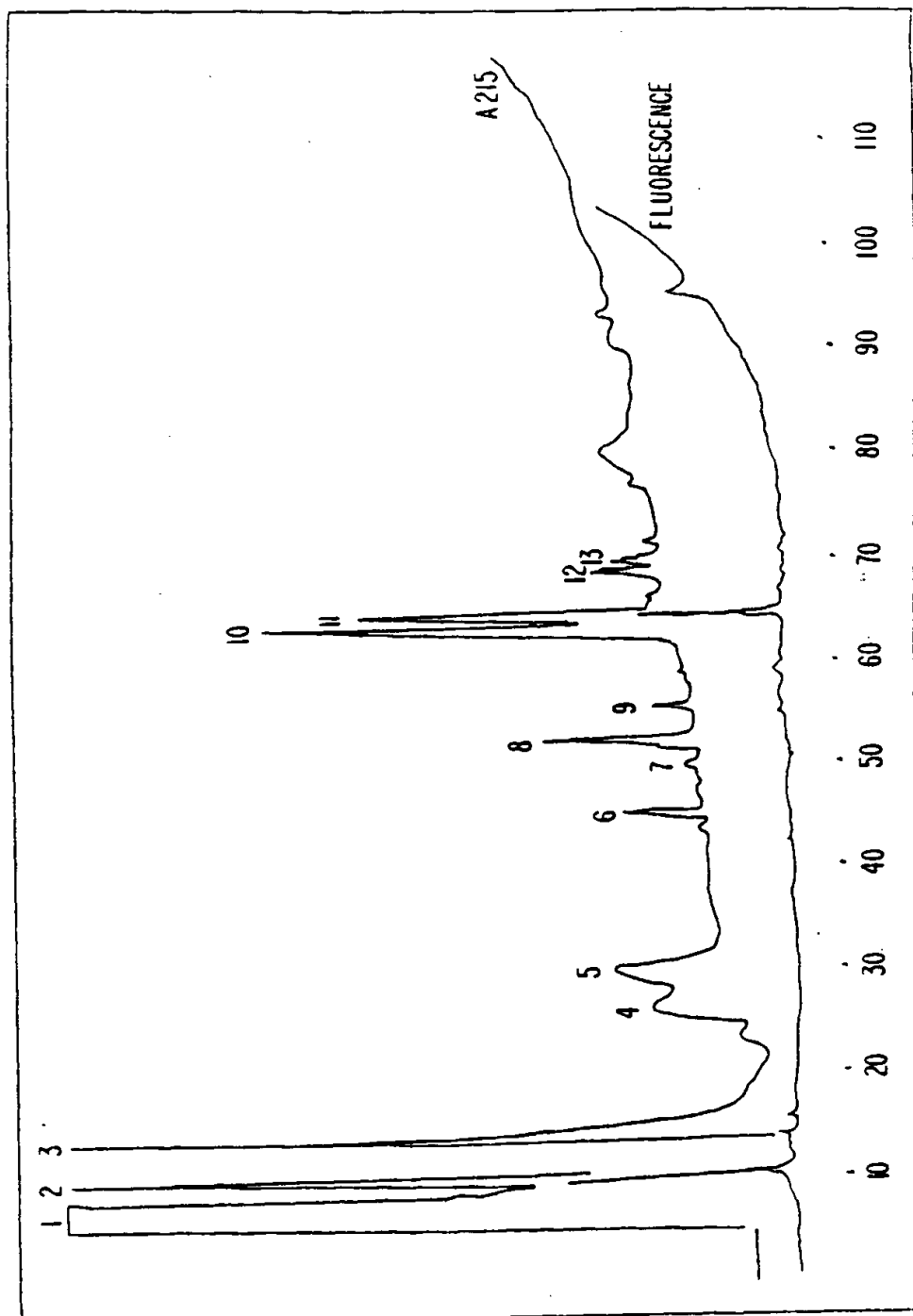
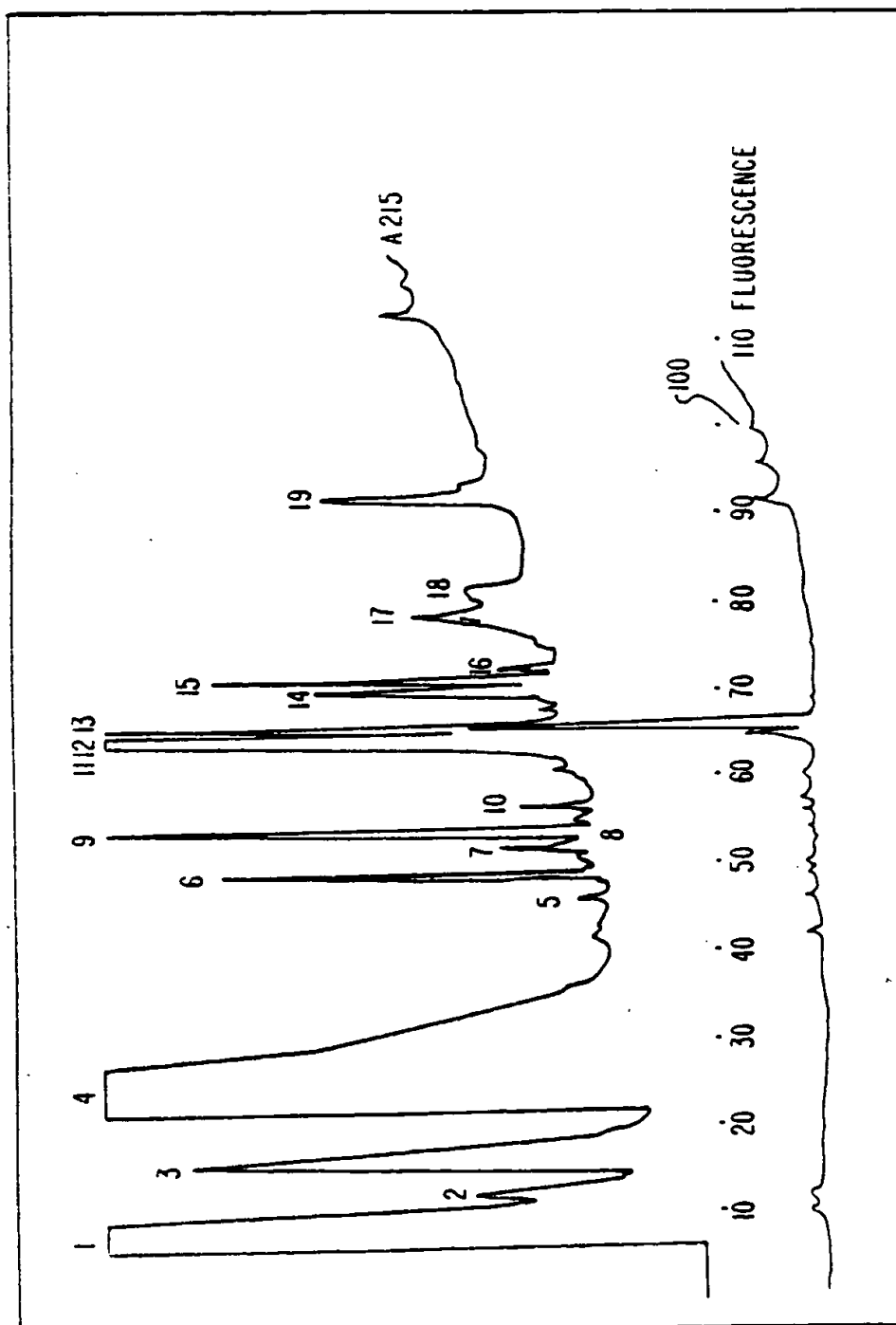


FIG.11B



40

20

60

A. D S V C P Q G K Y I H P Q N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D

\* \* \* \* \*

\* C \_ \_ \_ S G S F T A S E N H L R H C L S C S \_ \_ \_ K

B. (E) K Q N T V C T C H A G F F L R E N E C V S C (L) N C (K) (K) (C) (L) (K) (E)

\* \* \* \* \*

C. (K) E M G Q V E I S S C T V D R D T V C G C R K N (C) (Y) (R) (H) (Y)

\* \* \* \* \*

D. (K) (S) L E C T K L C L P Q I E N

(D)

## FIG. 13

CATGCTGCA GGTGACTGT AGAGGATCTG GGGCTACTA GCTTGAGTT GAGGGAAACA AATGAACAC  
 30 90 100 110 120 130 140  
 ACAGGACAAAG GAGAGAACA AAGGATCA GATTGATGC CCCAAGTGT TAAATTTCAA GGAAGAACTC  
 150 160 170 180 190 200 210  
 TAAACTTAGT GAGTGGCTG GCTGGGCGG AATGTTTAC TGAGGAAGGA CTGAGCCAG GGAAGTTTAA  
 220 230 240 250 260 270 280  
 GATCTGTAC CCTAAGCTT CCCATCCCTC CTTCTCTGA TGGTGCTCC TGTATCTGT TCTTCCCTAG  
 289 299 307 316 325 334  
 GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC  
 Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val  
 343 352 361 370 379 388  
 CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT  
 Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr  
 397 406 415 424 433 444  
 ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA G GTAGGGCAA  
 Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Ala 504 514  
 454 464 474 484 494  
 GTGGAAACGG TGAATGCCCT CAGGTCTGGG GTGTGTCTC TTCTCTGCT TCTTCCAGTT GTTCTTCCCT  
 524 534 544 554 564 574 584  
 AACTTTGTG TCTCTCTGG GCTGGGATT TCTCCCTCCC TCTTCTCTA GAGACTTCAG GGAATCCGCC  
 594 604 614 624 634 644 654  
 CTGGCTGTG TCCATAGCAT GGGGCTCCTT CTTGTGTTC TCACCCGCAG CCTAAGCTG CGGCCCAT  
 664 673 682 691 700  
 CA CA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC  
 Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp  
 709 718 727 736 745 754  
 TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC  
 Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His  
 763 772 781 797 807 817  
 TGC CTC AGC TGC TCC AAA TGC CCA AAG GGTGAGTGT CACAGGCAGG AGAGTCAGG  
 Cys Leu Ser Cys Ser Lys Cys Arg Lys  
 827 837 847 857 867 877 887  
 GGGCTTTGAG TGGTGTGG GTGCTGTCT ATGTGAGGC TGGTGGTGT GGGCAGGAAG GTGTGTGTT  
 897 907 917 927 937 947 957  
 TGGTGGGACA CTGCATGGAT GTGAGTGTGT ATTACAGAGA CACACACTTA GGGGTATGTC AGGAAGGGGA  
 967 977 987 997 1007 1016  
 TGCAGGGACA GGAGGATGCA GGAATCATAC CCTATCTCT CCCCTACCA GAA ATG GGT CAG  
 Glu MET Gly Gln  
 1025  
 GTC GAG ATC  
 Val Glu Ile

FIG. 14

1 10 20 30 40 50 60 70 80  
 DSVCFQGV IHPQNN SICCIKCHKGTYLYNOCPPGQDTCRECECESGSFIASENHLRHCLSCSKCRKEMGOVEISSCTIVDROT  
 90  
 VCGCRKN  
 KONTVCTCHAGFFLRENECVSC  
 IECTKLCIPQIEN

FIG. 15



FIG. 16

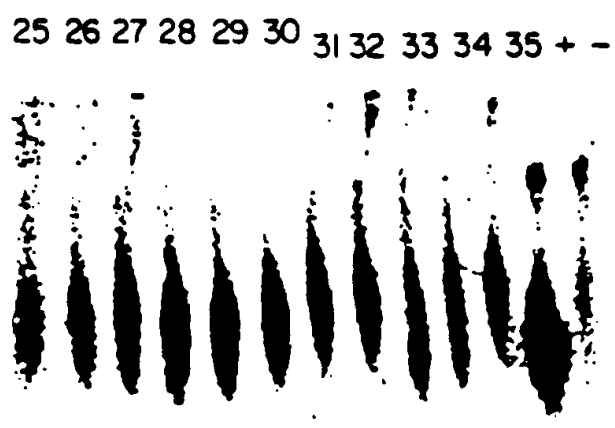


FIG.17

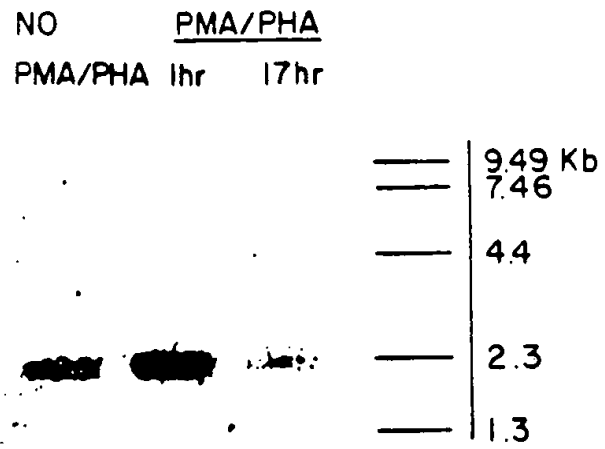


FIG.18

11 21 51 12 22 52



## FIG.19

Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr 20  
 Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp 40  
 Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu 60  
 Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp 80  
 Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu 100  
 Phe Gln Cys Phe Asn Cys Ser Leu Cys Ser Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu 120  
 Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Leu Arg Glu Asn Glu Cys Val 140  
 Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu 160  
 Asn



|  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |  |     |  |
|--|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|--|-----|--|
| 296  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 305 |     |  |  |     |  |
| CAT AGT GTC TGT CCC CAA<br>Asp Ser Val Cys Pro Gln |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |  |  |     |  |
| 314  |     |     |     | 323 |     |     |     | 332 |     |     |     | 341 |     |     |     | 350 |     |  |  | 359 |  |
| GGA  | AAA | TAT | ATC | CAC | CCT | CAA | AAT | AAT | TGC | ATT | TGC | TGT | ACC | AAG | TGC | CAC | AAA |  |  |     |  |
| Gly  | Lys | Tyr | Ile | His | Pro | Gln | Asn | Asn | Ser | Ile | Cys | Cys | Thr | Lys | Cys | His | Lys |  |  |     |  |
| 368  |     |     |     | 377 |     |     |     | 386 |     |     |     | 395 |     |     |     | 404 |     |  |  | 413 |  |
| GGA  | ACC | TAC | TTG | TAC | AAT | GAC | TGT | CCA | GGC | CCG | GGG | CAG | GAT | ACG | GAC | TGC | AGG |  |  |     |  |
| Gly  | Thr | Tyr | Leu | Tyr | Asn | Asp | Cys | Pro | Gly | Pro | Gly | Gln | Asp | Thr | Asp | Cys | Arg |  |  |     |  |
| 422  |     |     |     | 431 |     |     |     | 440 |     |     |     | 449 |     |     |     | 458 |     |  |  | 467 |  |
| GAG  | TGT | GAG | AGC | GGC | TCC | TTC | ACC | GCT | TCA | GAA | AAC | CAC | CTC | AGA | CAC | TGC | CTC |  |  |     |  |
| Glu  | Cys | Glu | Ser | Gly | Ser | Phe | Thr | Ala | Ser | Glu | Asn | His | Leu | Arg | His | Cys | Leu |  |  |     |  |
| 476  |     |     |     | 485 |     |     |     | 494 |     |     |     | 503 |     |     |     | 512 |     |  |  | 521 |  |
| AGC  | TGC | TCC | AAA | TGC | CGA | AAG | GAA | ATG | GGT | CAG | GTG | GAG | ATC | TCT | TCT | TGC | ACA |  |  |     |  |
| Ser  | Cys | Ser | Lys | Cys | Arg | Lys | Glu | MET | Gly | Gln | Val | Glu | Ile | Ser | Ser | Cys | Thr |  |  |     |  |
| 530  |     |     |     | 539 |     |     |     | 548 |     |     |     | 557 |     |     |     | 566 |     |  |  | 575 |  |
| GTG  | GAC | CGG | GAC | ACC | GTG | TGT | GGC | TGC | AGG | AAG | AAC | CAG | TAC | CGG | CAT | TAT | TGG |  |  |     |  |
| Val  | Asp | Arg | Asp | Thr | Val | Cys | Gly | Cys | Arg | Lys | Asn | Gln | Tyr | Arg | His | Tyr | Trp |  |  |     |  |
| 584  |     |     |     | 593 |     |     |     | 602 |     |     |     | 611 |     |     |     | 620 |     |  |  | 629 |  |
| AGT  | GAA | AAC | CTT | TTC | CAG | TGC | TTC | AAT | TGC | AGC | CTC | TGC | CTC | AAT | GGG | ACC | GTG |  |  |     |  |
| Ser  | Glu | Asn | Leu | Phe | Gln | Cys | Phe | Asn | Cys | Ser | Leu | Cys | Leu | Asn | Gly | Thr | Val |  |  |     |  |
| 638  |     |     |     | 647 |     |     |     | 656 |     |     |     | 665 |     |     |     | 674 |     |  |  | 683 |  |
| CAC  | CTC | TCC | TGC | CAG | GAG | AAA | CAG | AAC | ACC | GTG | TGC | ACC | TGC | CAT | GCA | GGT | TTC |  |  |     |  |
| His  | Leu | Ser | Cys | Gln | Glu | Lys | Gln | Asn | Thr | Val | Cys | Thr | Cys | His | Ala | Gly | Phe |  |  |     |  |
| 692  |     |     |     | 701 |     |     |     | 710 |     |     |     | 719 |     |     |     | 728 |     |  |  | 737 |  |
| TTT  | CTA | AGA | GAA | AAC | GAG | TGT | GTC | TCC | TGT | AGT | AAC | TGT | AAC | AAA | AGC | CTG | GAG |  |  |     |  |
| Phe  | Leu | Arg | Glu | Asn | Glu | Cys | Val | Ser | Cys | Ser | Asn | Cys | Lys | Lys | Ser | Leu | Glu |  |  |     |  |
| 746  |     |     |     | 755 |     |     |     | 764 |     |     |     |     |     |     |     |     |     |  |  |     |  |
| TGC  | ACG | AAG | TTG | TGC | CTA | CCC | CAG | ATT | GAG | AAT |     |     |     |     |     |     |     |  |  |     |  |
| Cys  | Thr | Lys | Leu | Cys | Leu | Pro | Gln | Ile | Glu | Asn |     |     |     |     |     |     |     |  |  |     |  |

## FIG. 21A

|   |     |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|-----|
| 10  | 20  | 30  | 40  | 50  | 60  | 70  |
| GATCACTGGG ACCAGGGGT GATCTCTATG CCGCAGTCTC ACCCTCACC TGTACACCA AGGCACCTTG   |     |     |     |     |     |     |
| 80  | 90  | 100 | 110 | 120 | 130 | 140 |
| GACGTCTGG ACAGACCGAG TCCCGGGAG CCCCAGCACT GCCGCTGCCA CACTGCCCTG AGCCCAATG   |     |     |     |     |     |     |
| 150   | 160 | 171 | 180 | 189 | 198 |     |
| GGGGAGTGAG AGGCCATAGC TGCTGGC ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG       |     |     |     |     |     |     |
| MET Gly Leu Ser Thr Val Pro Asp Leu Leu                                     |     |     |     |     |     |     |
| 207   | 216 | 225 | 234 | 243 | 252 |     |
| CTG CCG CTG CTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT     |     |     |     |     |     |     |
| Leu Pro Leu Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile     |     |     |     |     |     |     |
| 261   | 270 | 279 | 288 | 297 | 306 |     |
| GSA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA     |     |     |     |     |     |     |
| Gly Leu Val Pro His Leu Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln |     |     |     |     |     |     |
| 315   | 324 | 333 | 342 | 351 | 360 |     |
| GSA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA     |     |     |     |     |     |     |
| Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys     |     |     |     |     |     |     |

FIG. 21B

|   |   |     |     |     |     |
|---|---|-----|-----|-----|-----|
| 369   | 378   | 387 | 396 | 405 | 414 |
| GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG         | Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg         |     |     |     |     |
| 423   | 432   | 441 | 450 | 459 | 468 |
| GAG TGT GAG AGC TTC TCC GGC TCC AAG GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC     | Glu Cys Glu Ser Gly Ser Phe Thr ACC Thr Ala Ser Glu Asn His Leu Arg His Cys Leu |     |     |     |     |
| 477   | 486   | 495 | 504 | 513 | 522 |
| TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA             | Ser Cys Ser Lys Cys Cys Arg Lys Glu MET Gly Gln Val Glu Ile Ser Ser Cys Thr     |     |     |     |     |
| 531   | 540   | 549 | 558 | 567 | 576 |
| GTC GAC CGG GAC ACC ACC GTG TGT GGC TGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG | Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp         |     |     |     |     |
| 583   | 594   | 603 | 612 | 621 | 630 |
| AGT GAA AAC CTT TTC CAG TTC AAT TGC AGC CTC TGC CTC AAT AAT GGG ACC GTG         | Ser Glu Asn Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val     |     |     |     |     |
| 639   | 648   | 657 | 666 | 675 | 684 |
| CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC         | His Leu Ser Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe     |     |     |     |     |

FIG.21C

| 693  | 702 | 711 | 720 | 729 | 738  |
|--|-----|-----|-----|-----|------|
| TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG<br>Phe Leu Arg Glu Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu     | 747 | 750 | 765 | 774 | 783  |
| TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA<br>Cys Thr Lys Leu Cys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser | 801 | 810 | 819 | 828 | 837  |
| GGC ACC ACA GTC CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC<br>Gly Thr Thr Val Val Leu Leu Pro Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu Ser | 853 | 864 | 873 | 882 | 891  |
| CTC CTC TTC ATT GGT TTA ATG TAT TAC CAA CGG TGG AAG TCC AAG CTC TAC<br>Leu Leu Phe Ile Gly Leu Leu MET Tyr Arg Tyr Gln Arg Trp Lys Ser Lys Leu Tyr         | 909 | 918 | 927 | 936 | 945  |
| TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT<br>Ser Ile Val Val Cys Gly Lys Ser Thr Thr Pro Pro Glu Lys Glu Gly Glu Leu Gly Thr | 963 | 972 | 981 | 990 | 999  |
| ACT ACT AAG CCC CTG GCC CCA AAC CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC<br>Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr         |     |     |     |     | 1008 |

FIG. 21D

|   |   |      |      |      |      |
|---|---|------|------|------|------|
| 1017  | 1026  | 1035 | 1044 | 1053 | 1062 |
| ACC CTG GGC TTC AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC ACC                 | Pro Thr Leu Gly Phe Ser Pro Val Pro Ser Thr Phe Thr Ser Ser Thr             |      |      |      |      |
| 1071  | 1080  | 1089 | 1098 | 1107 | 1116 |
| TAT ACC CCC GGT GAC TGT TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA CCA | Tyr Thr Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro     |      |      |      |      |
| 1125  | 1134  | 1143 | 1152 | 1161 | 1170 |
| CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC TCC GAC CCC ATC     | Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile     |      |      |      |      |
| 1179  | 1188  | 1197 | 1206 | 1215 | 1224 |
| CCC AAC CCC CTT CAG AAG TGG GAG GAC AGC GCG CAC AAG CCA CAG AGC CTA GAC     | Pro Asn Pro Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp |      |      |      |      |
| 1233  | 1242  | 1251 | 1260 | 1269 | 1278 |
| ACT GAT GAC CCC GCG ACG CTG TAC GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC     | Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro Leu Arg     |      |      |      |      |
| 1287  | 1296  | 1305 | 1314 | 1323 | 1332 |
| TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG ATC GAT CGG CTG     | Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp Arg Leu     |      |      |      |      |

FIG. 21E

|   |      |      |      |      |      |
|---|------|------|------|------|------|
| 1341  | 1350 | 1359 | 1368 | 1377 | 1386 |
| CTG CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC                 |      |      |      |      |      |
| Glu Leu Gln Asn Gly Arg Cys Leu Arg Cys Leu Arg Glu Ala Gln Tyr Ser MET Leu Ala Thr |      |      |      |      |      |
| 1395  | 1404 | 1413 | 1422 | 1431 | 1440 |
| GG AGG CGG CGC ACG CCG CGG CGC GAG GCG CAA TAC AGC ATG CTG GCG GTG                  |      |      |      |      |      |
| Arg Arg Arg Arg Thr Pro Arg Arg Arg Glu Ala Thr Leu Glu Leu Leu Gly Arg Val         |      |      |      |      |      |
| 1449  | 1458 | 1467 | 1476 | 1485 | 1494 |
| CTC CGC GAC ATG GAC CTG CTG GGC TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC             |      |      |      |      |      |
| Leu Arg Asp MET Asp Leu Leu Leu Gly Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys         |      |      |      |      |      |
| 1503  | 1512 | 1521 | 1530 | 1546 | 1556 |
| GG CCC GCC GGC CTC CCG CGC GCG CCC AGT CTT CTC AGA TGA GGC TGGCCCC CTGCGGGCAG       |      |      |      |      |      |
| Gly Pro Ala Ala Leu Pro Pro Ala Pro Ser Leu Leu Arg                                 |      |      |      |      |      |
| 1566  | 1576 | 1586 | 1596 | 1606 | 1616 |
| CTCTAAGGAC CGTCCTGCCA GATCGCCCTC CAACCCCACT TTTTCTGG AAGGAGGGGT CCTGCAGGGG          |      |      |      |      |      |
| 1636  | 1646 | 1656 | 1666 | 1676 | 1686 |
| CAAGCAGGAG CTAGCAGCG CCTACTTGGT GCTAACCCCT CGATGTACAT AGCTTTCTC AGCTGCTGC           |      |      |      |      |      |
| 1696  |      |      |      |      |      |

FIG.21F

|            |            |             |            |            |             |             |
|------------|------------|-------------|------------|------------|-------------|-------------|
| 1706       | 1716       | 1726        | 1736       | 1746       | 1756        | 1766        |
| GGCGCGCCGA | CAGTCAGCGC | TGTGGCGCGG  | GAGAGAGGTG | CGCCGTGGGC | TCAAGAGCCT  | GAGTGGGTGG  |
| 1776       | 1786       | 1796        | 1806       | 1816       | 1826        | 1836        |
| TTTGGGAGGA | TGAGGGAGCG | TATGCCCTCAT | GCCCGTTTTG | GGTGTCCTCA | CCAGCAAGGC  | TGCTCGGGGG  |
| 1846       | 1856       | 1866        | 1876       | 1886       | 1896        | 1906        |
| CCCCTGGTTC | GTCCCTGAGC | CTTTTTCACA  | GTCATAAGC  | AGTTTTTTTT | GTTTTTGTTT  | TGTTTTGTTT  |
| 1916       | 1926       | 1936        | 1946       | 1956       | 1966        | 1976        |
| TGTTTTTAAA | TCATCAIGT  | TACACTAATA  | GAAACTTGGC | ACTCCTGTGC | CCYCTGCCCTG | GACAAGGCACA |
| 1986       | 1996       | 2006        | 2016       | 2026       | 2036        | 2046        |
| TAGCAAGCTG | AACGTGCTTA | AGGCAGGGGC  | GAGCACGGAA | CAATGGGGCC | TTCAGCTGGA  | GCTGTGGACT  |
| 2056       | 2066       | 2076        | 2086       |            |             |             |
| TTTGTACATA | CACIAAAATT | CTGAAGTTAA  | AGCTCAAAAA | AA         |             |             |

## FIG.22

GA ATT CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT

Start gene 10 protein sequence

ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT ACG GAT CCG ATC TTG GAG GAT CAT TAA  
Met Ala Ser Met Thr Gly Gly Gln Met Gly Thr Asp Pro Ile Leu Glu Asp Asp stop  
Translational coupler

ATG GAC ACC GTT TCC CCC  
Met Asp Ser Val Cys Pro  
Start TNF inhibitor sequence



FIG. 23

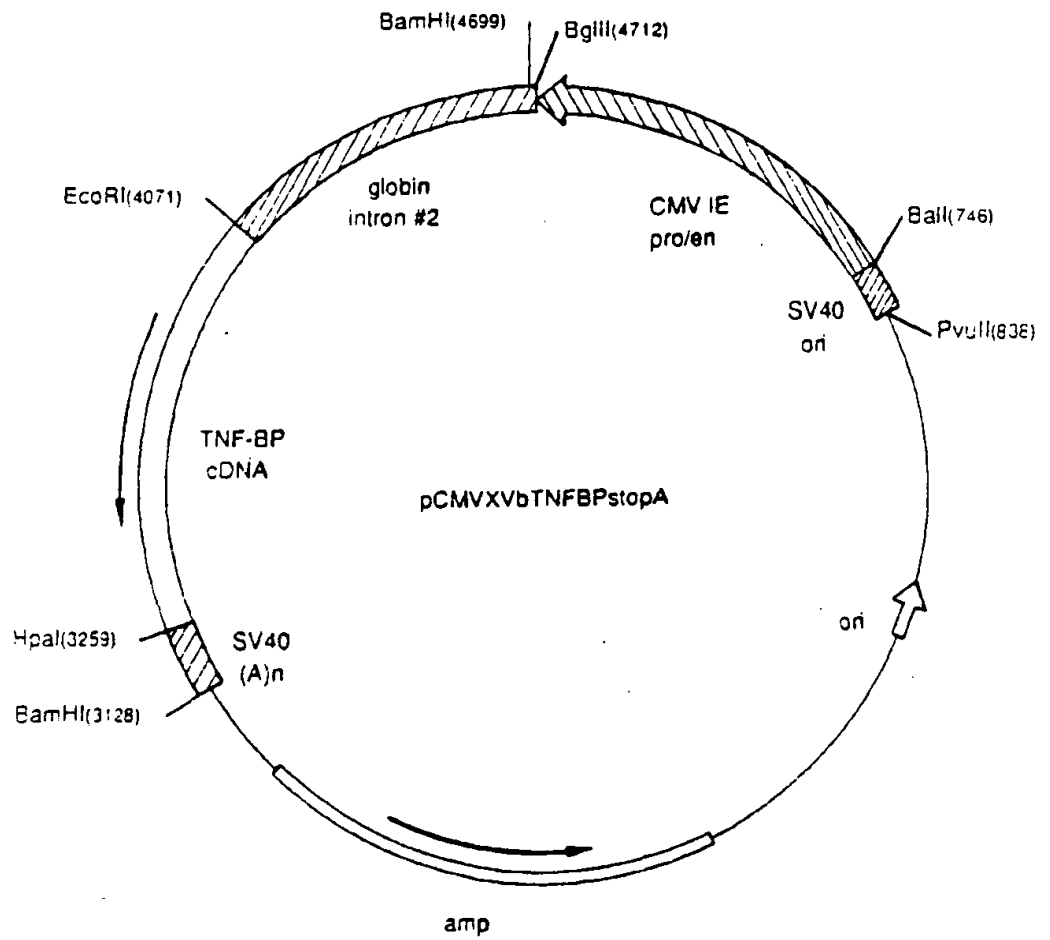


FIG. 24

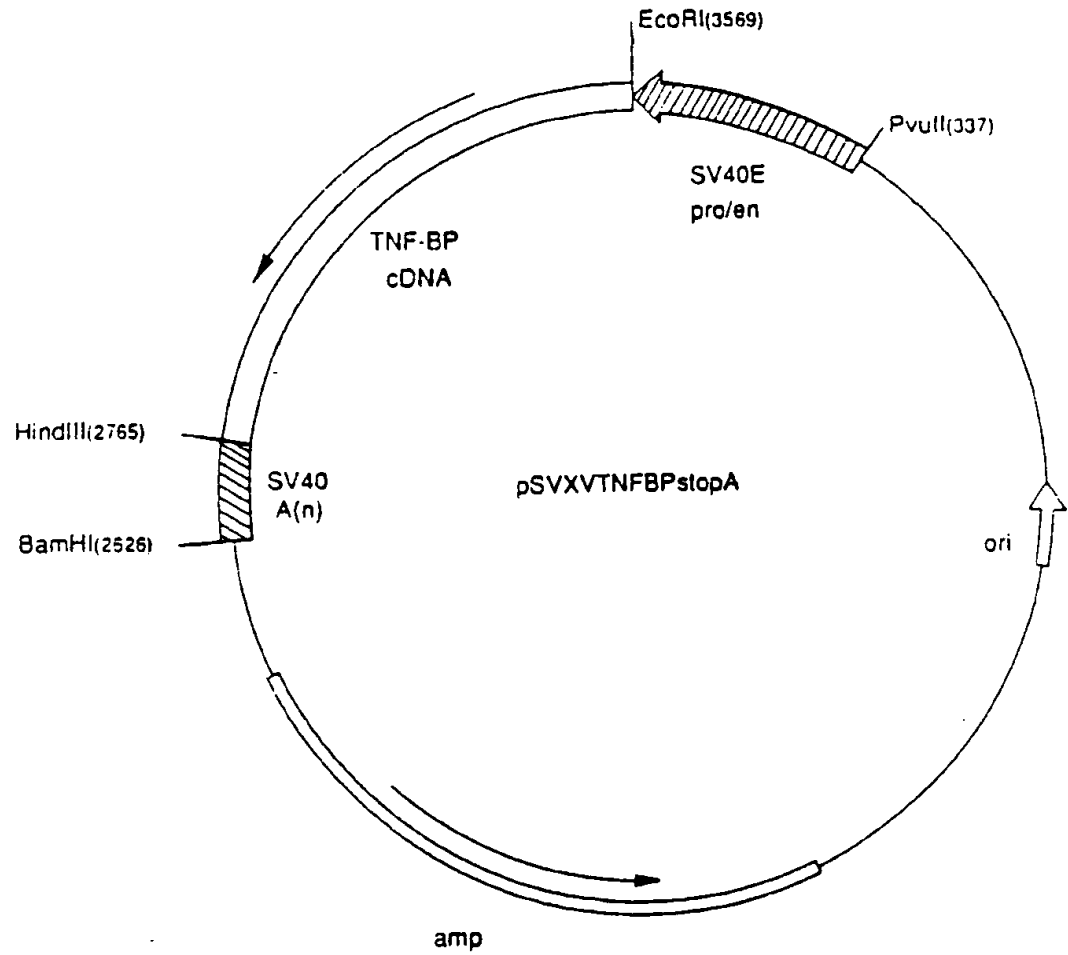


FIG. 25

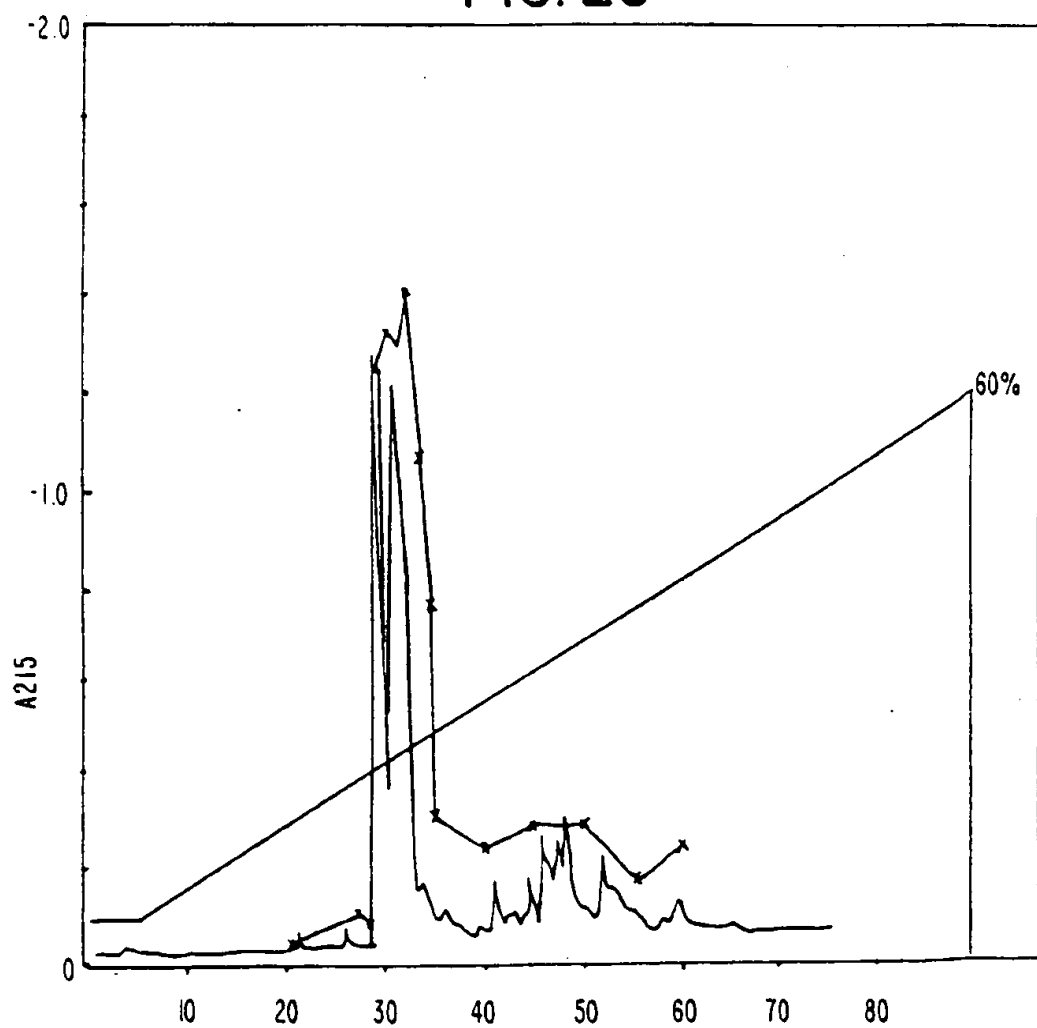


FIG.26



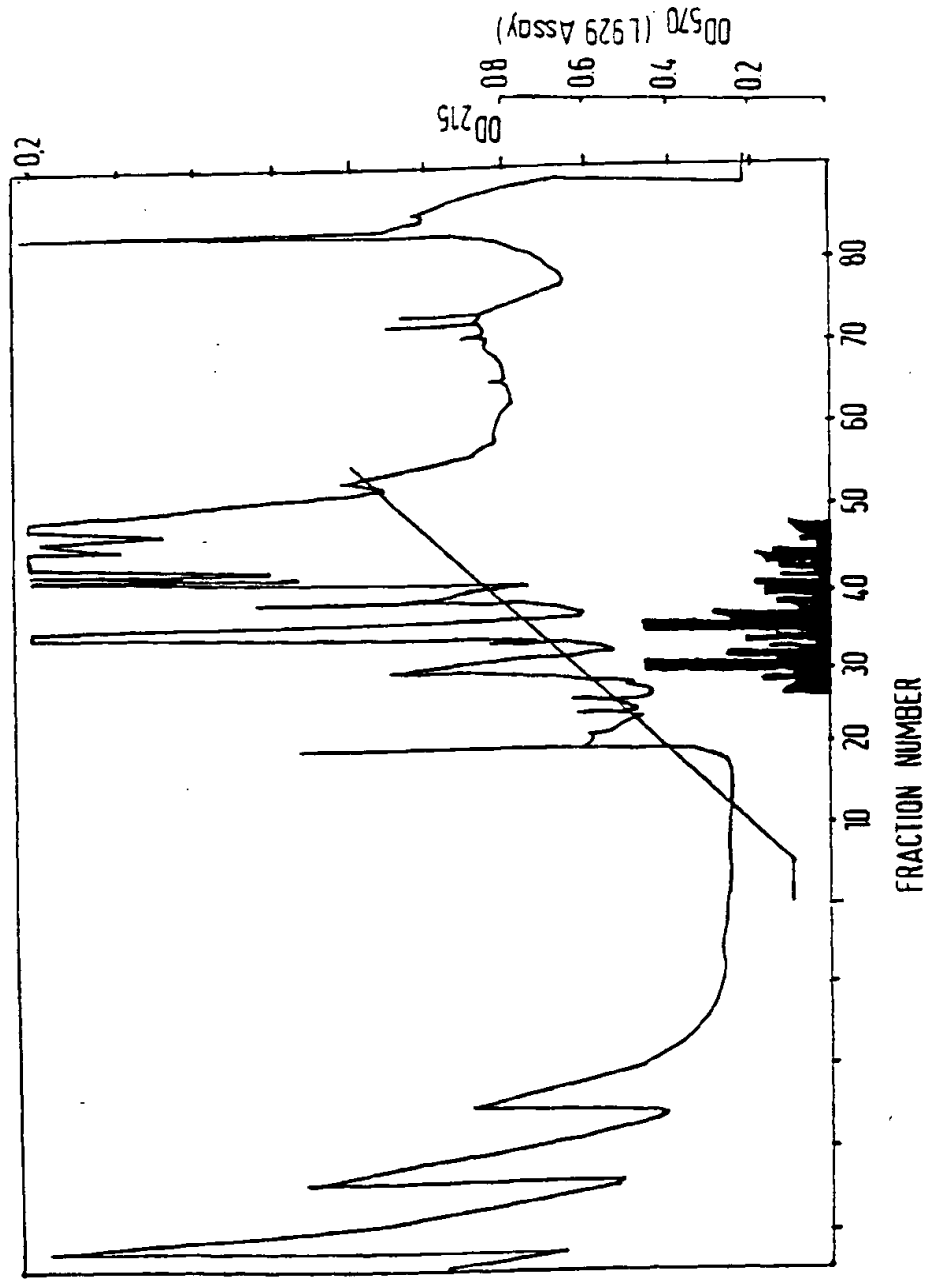


FIG. 27

FIG. 28

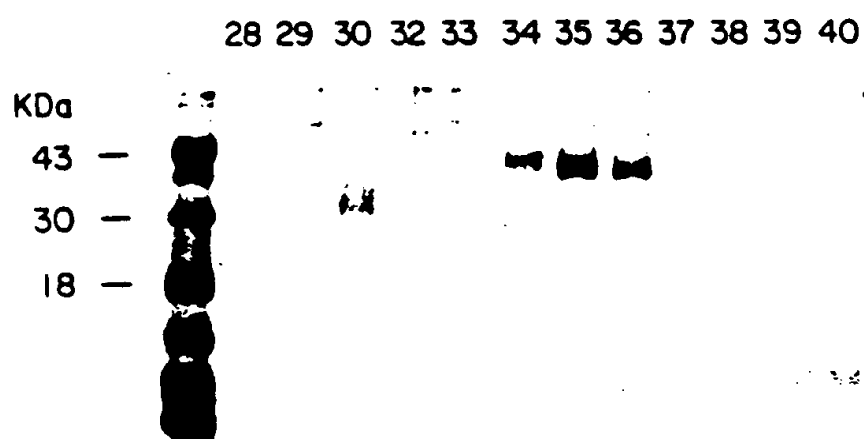


FIG. 29

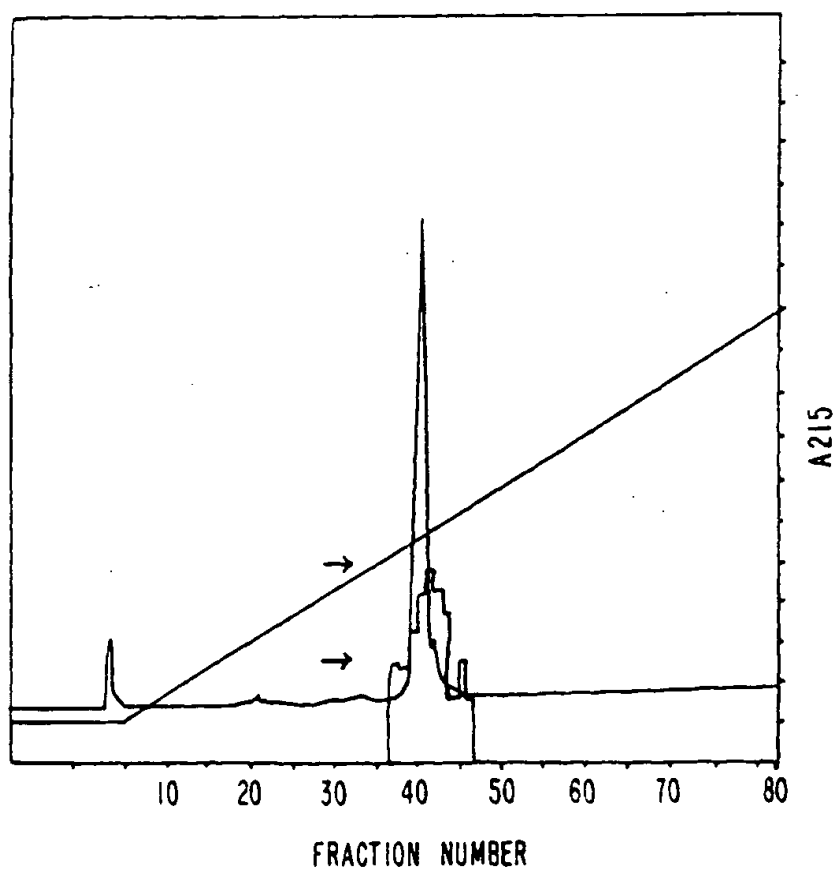


FIG.30

34 33 32 31 30 29



- 43 kDa  
- 30 kDa  
- 18 kDa



## FIG.3I

U937-derived TNF inhibitor1 (30 kDa)

( )-( )-Val-( )-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-( )-Asn-( )-Ile-

U937-derived TNF inhibitor2 (40 kDa)

Leu-Pro-Ala-Gln-Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys-Arg-

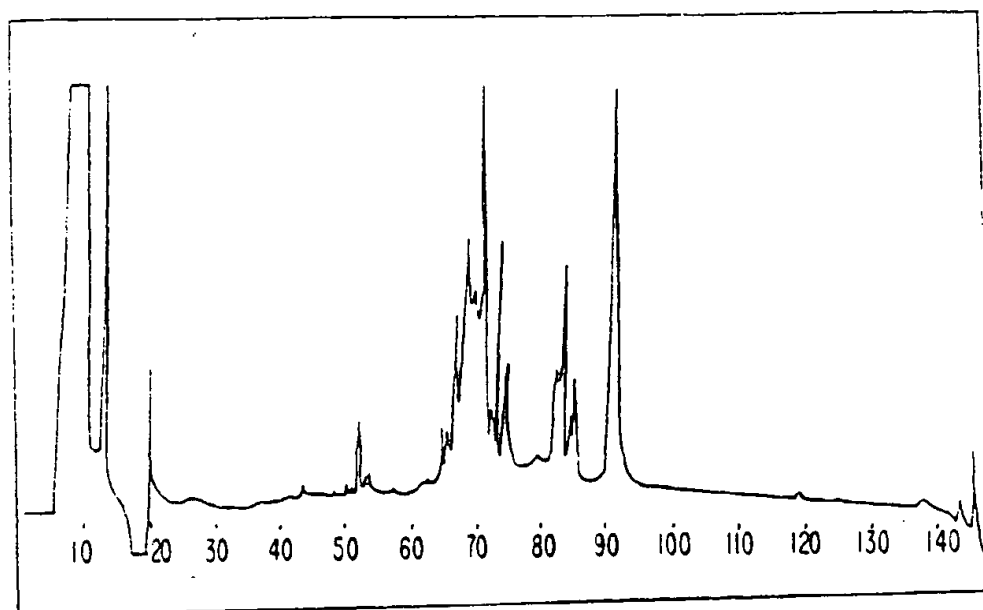
Leu-Arg-Glu-Tyr-Tyr-Asp-Gln-Thr-Ala-Gln-Met-Cys-Cys-Ser-Lys-Cys-

Urine-derived TNF inhibitor2 (40 kDa)

Ala-Gln-Val-Ala-Phe-Thr-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys-( )-Leu-( )-

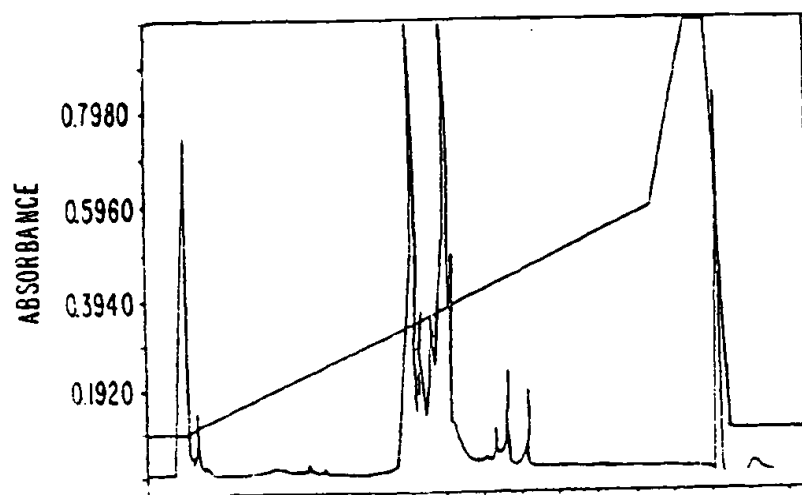
Glu-

FIG. 32



V8 DIGEST

FIG. 33



Arg. C A215

FIG. 34

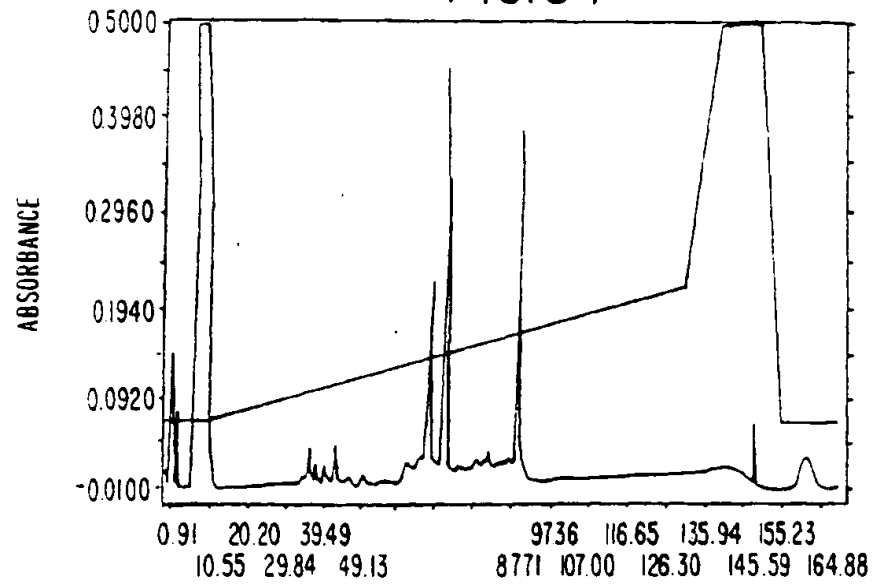


FIG. 35

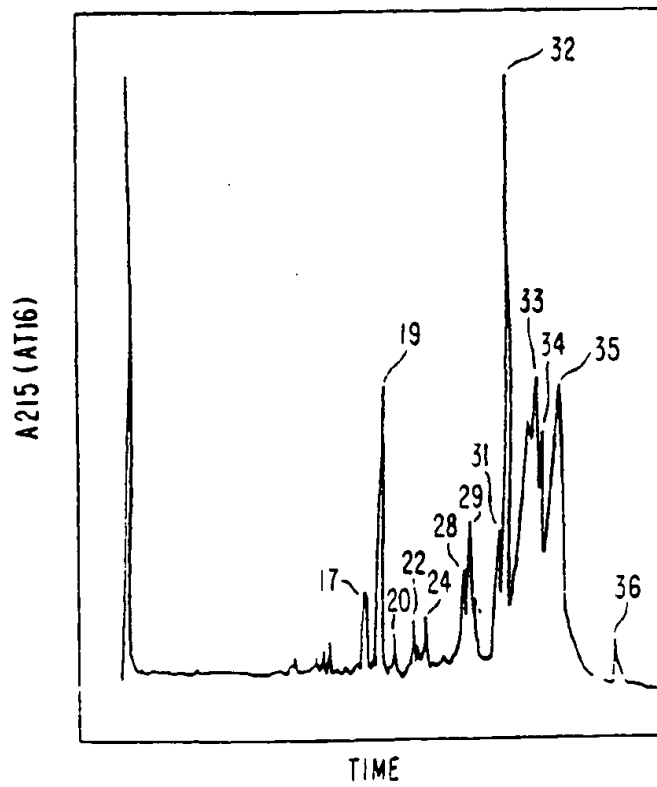
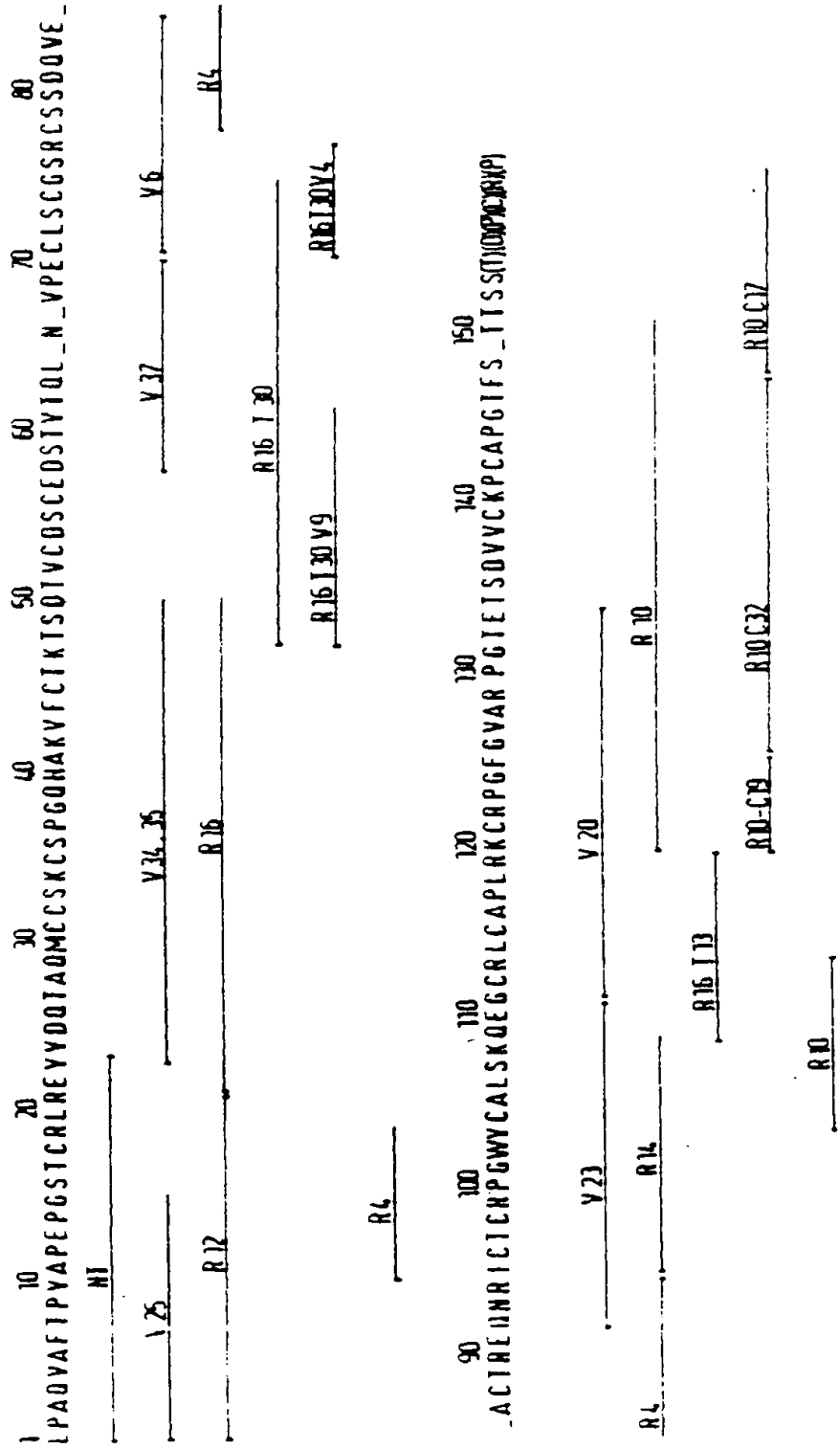


FIG.36



|            |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |               |  |  |     |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---------------|--|--|-----|
|            |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 5'-CCG<br>Pro |  |  |     |
| 58         |     |     |     | 64  |     |     |     | 73  |     |     |     | 62  |     |     |     | 91  |               |  |  | 100 |
| GAG        | CCC | GGG | AGC | ACA | TGC | CGG | CTC | AGA | GAA | TAC | TAT | GAC | CAG | ACA | GCT | CAG | ATG           |  |  |     |
| Glu        | Pro | Gly | Ser | Thr | Cys | Arg | Leu | Arg | Glu | Tyr | Tyr | Asp | Gln | Thr | Ala | Gln | MET           |  |  |     |
| 109        |     |     |     | 118 |     |     |     | 127 |     |     |     | 136 |     |     |     | 145 |               |  |  | 154 |
| TGC        | TGC | AGC | AAG | TGC | TGC | CCG | GGC | CAA | CAT | GCA | AAA | GTC | TTC | TGT | ACC | AAG | ACC           |  |  |     |
| Cys        | Cys | Ser | Lys | Cys | Ser | Pro | Gly | Gln | His | Ala | Lys | Val | Phe | Cys | Thr | Lys | Thr           |  |  |     |
| 63         |     |     |     | 172 |     |     |     | 181 |     |     |     | 190 |     |     |     | 199 |               |  |  | 208 |
| TCC        | GAC | ACC | GTG | TGT | GAC | TCC | TGT | GAG | GAC | AGC | ACA | TAC | ACC | CAG | CTC | TGG | AAC           |  |  |     |
| Ser        | Asp | Thr | Val | Cys | Asp | Ser | Cys | Glu | Asp | Ser | Thr | Tyr | Thr | Gln | Leu | Trp | Asn           |  |  |     |
| 217        |     |     |     | 226 |     |     |     | 235 |     |     |     | 244 |     |     |     | 253 |               |  |  | 262 |
| TGG        | GTT | CCC | GAG | TGC | TTG | AGC | TGT | GGC | TCC | CGC | TGT | AGC | TCT | GAC | CAG | GTG | GAA           |  |  |     |
| Trp        | Val | Pro | Glu | Cys | Leu | Ser | Cys | Gly | Ser | Arg | Cys | Ser | Ser | Asp | Gln | Val | Glu           |  |  |     |
| 217        |     |     |     | 280 |     |     |     | 289 |     |     |     | 298 |     |     |     | 307 |               |  |  | 316 |
| ACT        | CAA | GCC | TGC | ACT | CGG | GAA | CAG | AAC | CGC | ATC | TGC | ACC | TGC | AGG | CCC | GGC | TGG           |  |  |     |
| Thr        | Gln | Ala | Cys | Thr | Arg | Glu | Gln | Asn | Arg | Ile | Cys | Thr | Cys | Arg | Pro | Gly | Trp           |  |  |     |
| 325        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |               |  |  |     |
| TAC TGC-3' |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |               |  |  |     |
| Tyr Cys    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |               |  |  |     |

FIG. 37

## FIG.38

Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr Ala Pro Glu Pro Gly Ser  
Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln Thr Ala Gln Met Cys Cys  
Ser Lys Cys Ser Pro Gly Gln His Ala Lys Val Phe Cys Thr Lys Thr  
Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu  
Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser  
Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys  
Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys  
Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala  
Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro  
Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His  
Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala  
Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val  
His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Thr  
Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly  
Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp

10 20 30 40 50 60 70  
 GAATTCGGCG CAGCGGAGCC TGGAGAGAAG GCGCTGGGCT GCGAGGGGCG GAGGGGCGGA GGGCAGGGGG

80 90 101 110 119  
 CAACCGGACC CCGCCCGCAC CC ATG GCG CCC GTC GCG GTC TGG GCG GCG CTG GCG  
 MET Ala Pro Val Ala Val Trp Ala Ala Leu Ala

128 137 146 155 164 173  
 GTC GGA CTG GAG CTC TGG GCT GCG GCG CAC GCG TTG CCC GCG CAG GTG GCA TTT  
 Val Gly Leu Glu Leu Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe

182 191 200 209 218 227  
 ACA CCC TAC GCC CCG GAG CCC GGG AGC ACA TGC CCG CTC AGA GAA TAC TAT GAC  
 Thr Pro Tyr Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp

236 245 254 263 272 281  
 CAG ACA GCT CAG ATG TGC TGC AGC AAG TGC TCG CCG GGC CAA CAT GCA AAA GTC  
 Gln Thr Ala Gln MET Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys Val

290 299 308 317 326 335  
 TTC TGT ACC AAG ACC TCG GAC ACC GTG TGT GAC TCC TGT GAG GAC AGC ACA TAC  
 Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr

344 353 362 371 380 389  
 ACC CAG CTC TGG AAC TGG GTT CCC GAG TGC TTG AGC TGT GGC TCC CGC TGT AGC  
 Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser

398 407 416 425 434 443  
 TCT GAC CAG GTG GAA ACT CAA GCC TGC ACT CCG GAA CAG AAC CGC ATC TGC ACC  
 Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr

452 461 470 479 488 497  
 TGC AAG CCC GGC TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC CCG CTG TGC  
 Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg Leu Cys

506 515 524 533 542 551  
 GCG CCG CTG CGC AAG TGC CCG CCG GGC TTC GGC GTG GCC AGA CCA GGA ACT GAA  
 Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu

560 569 578 587 596 605  
 ACA TCA GAC GTG GTG TGC AAG CCC TGT GCC CCG GCG ACB TTC TCC AAC ACB ACT  
 Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr

614 623 632 641 650 659  
 TCA TCC ACG GAT ATT TGC AGG CCC CAC CAG ATC TGT AAC GTG GTG GCC ATC CCT  
 Ser Ser Thr Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro

668 677 686 695 704 713  
 GCG AAT GCA AGC AGG GAT GCA GTC TGC ACG TCC ACB TCC CCC ACC CCG AGT ATG  
 Gly Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser MET

722 731 740 749 758 767  
 GCC CCA GCG GCA GTA CAC TTA CCC CAG CCA GTG TCC ACA CGA TCC CAA CAC ACG  
 Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr

776 785 794 803 812 821  
 CAG CCA ACT CCA GAA CCC AGC ACT GCT CCA AGC ACC TCC TTC CTG CTC CCA ATG  
 Gln Pro Thr Pro Glu Pro Ser Thr His Pro Ser Thr Ser Pro Leu Leu Pro Met

830 839 848 857 866 875  
 GGC CCC AGC CCC CCA GCT GAA GGG AGC ACT GGC GAC TTC GCT CTT CCA GTT GGA  
 Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val Gly

FIG. 39

|   |      |      |      |      |      |
|---|------|------|------|------|------|
| 884   | 893  | 902  | 911  | 920  | 929  |
| CTG ATT GTG GGT GTG ACA GCC TTG GGT CTA CTA ATA ATA GGA GTG GTG AAC TGT     |      |      |      |      |      |
| Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val Val Asn Cys     |      |      |      |      |      |
| 938   | 947  | 956  | 965  | 974  | 983  |
| GTC ATC ATG ACC CAG GTG AAA AAG AAG CCC TTG TGC CTG CAG AGA GAA GCC AAG     |      |      |      |      |      |
| Val Ile MET Thr Gln Val Lys Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys     |      |      |      |      |      |
| 992   | 1001 | 1010 | 1019 | 1028 | 1037 |
| GTG CCT CAC TTG CCT GCC GAT AAG GCC CGG GGT ACA CAG GGC CCC GAG CAG CAG     |      |      |      |      |      |
| Val Pro His Leu Pro Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu Gln Gln     |      |      |      |      |      |
| 1046  | 1055 | 1064 | 1073 | 1082 | 1091 |
| CAC CTG CTG ATC ACA GCG CCG AGC TCC AGC AGC AGC TCC CTG GAG AGC TCG GCC     |      |      |      |      |      |
| His Leu Leu Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala     |      |      |      |      |      |
| 1100  | 1109 | 1118 | 1127 | 1136 | 1145 |
| AGT GCG TTG GAC AGA AGG GCG CCC ACT CGG AAC CAG CCA CAG GCA CCA GGC GTG     |      |      |      |      |      |
| Ser Ala Leu Asp Arg Arg Ala Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val     |      |      |      |      |      |
| 1154  | 1163 | 1172 | 1181 | 1190 | 1199 |
| GAG GCC AGT GGG GCC GGG GAG GCC CGG GCC AGC ACC GGG AGC TCA GAT TCT TCC     |      |      |      |      |      |
| Glu Ala Ser Gly Ala Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser Asp Ser Ser     |      |      |      |      |      |
| 1208  | 1217 | 1226 | 1235 | 1244 | 1253 |
| CCT GGT GGC CAT GGG ACC CAG GTC AAT GTC ACC TGC ATC GTG AAC GTC TGT AGC     |      |      |      |      |      |
| Pro Gly Gly His Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser     |      |      |      |      |      |
| 1262  | 1271 | 1280 | 1289 | 1298 | 1307 |
| AGC TCT GAC CAC AGC TCA CAG TGC TCC TCC CAA GCC AGC TCC ACA ATG GGA GAC     |      |      |      |      |      |
| Ser Ser Asp His Ser Ser Gln Cys Ser Ser Ser Gln Ala Ser Ser Thr MET Gly Asp |      |      |      |      |      |
| 1316  | 1325 | 1334 | 1343 | 1352 | 1361 |
| ACA GAT TCC AGC CCC TCG GAG TCC CCG AAG GAC GAG CAG GTC CCC TTC TCC AAG     |      |      |      |      |      |
| Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val Pro Phe Ser Lys     |      |      |      |      |      |
| 1370  | 1379 | 1388 | 1397 | 1406 | 1415 |
| GAG GAA TGT GCC TTT CGG TCA CAG CTG GAG ACG CCA GAG ACC CTG CTG GGG AGC     |      |      |      |      |      |
| Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser     |      |      |      |      |      |
| 1424  | 1433 | 1442 | 1451 | 1460 | 1469 |
| ACC GAA GAG AAG CCC CTG CCC CTT GGA GTG CCT GAT GCT GGG ATG AAG CCC AGT     |      |      |      |      |      |
| Thr Glu Glu Lys Pro Leu Pro Leu Gly Val Pro Asp Ala Gly MET Lys Pro Ser     |      |      |      |      |      |

FIG. 39 continued



1473            1488            1498            1508            1518            1528            1538  
 >  
 TAA CCAGGCCCGT GTGGGCTGTG TCGTAGCCAA GGTGGGCTGA GCCCTGCCAG GATGACCCCTG  
 1548            1558            1568            1578            1588            1598            1608  
 CGAAGGGGGC CTGCTCCTTC CAGGCCCCCA CCACTAGGAC TCTGAGGCTC TTTCTGGGCC AAGTTCCTCT  
 1618            1628            1638            1648            1658            1668            1678  
 AGTGCCCTCC ACAGCCGCAG CCTCCCTCTG ACCTGCAGGC CAAGAGCAGA GGCAGCGGGT TGTGGAAAGC  
 1688            1698            1708            1718            1728            1738            1748  
 TTCTGCTGCC ATGGTGTGTC CCTCTCGGAA GGCTGGCTGG GCATGGACGT TCGGGGCATG CTGGGGCAAG  
 1758            1768            1778            1788            1898            1808            1818  
 TCCCTGACTC TCTGTGACCT GCCCCGCCCA GCTGCACCTG CCAGCCTGGC TTCTGGAGCC CTTGGGTTTT  
 1828            1838            1848            1858            1868            1878            1888  
 TTGTTTGTTC GTTTGTTTGT TTGTTTGTTC CTCCCCCTGG GCTCTGCCCC AGCTCTGGCT TCCAGAAAAC  
 1898            1908            1918            1928            1938            1948            1958  
 CCCAGCATCC TTTTCTGCAG AGGGGCTTTC TGGAGAGGAG GGATGCTGCC TGAGTCACCC ATGAAGACAG  
 1968            1978            1988            1998            2008            2018            2028  
 GACAGTGCTT CAGCCTGAGG CTGAGACTGC GGGATGGTCC TGGGGCTCTG TGCAGGGAGG AGGTGGCAGC  
 2038            2048            2058            2068            2078            2088            2098  
 CCTGTAGGGA ACGGGGTCCT TCAAGTTAGC TCAGGAGGCT TGGAAAGCAT CACCTCAGGC CACTGTGCCC  
 2108            2118            2128            2138            2148            2158            2168  
 ACCCCGATTT AAACCTTTTA TCTCCCAAAT GGGAAATATAA GAACCTGTCC TTTCTATCAC AAAGGGAGAT  
 2178            2188            2198            2208            2218  
 TGTGAGCAAG AGGGCAATTA ATAATAATGG CCAAATAATT AAAAAACCT AATTC

FIG. 39 CONT.

FIG.40

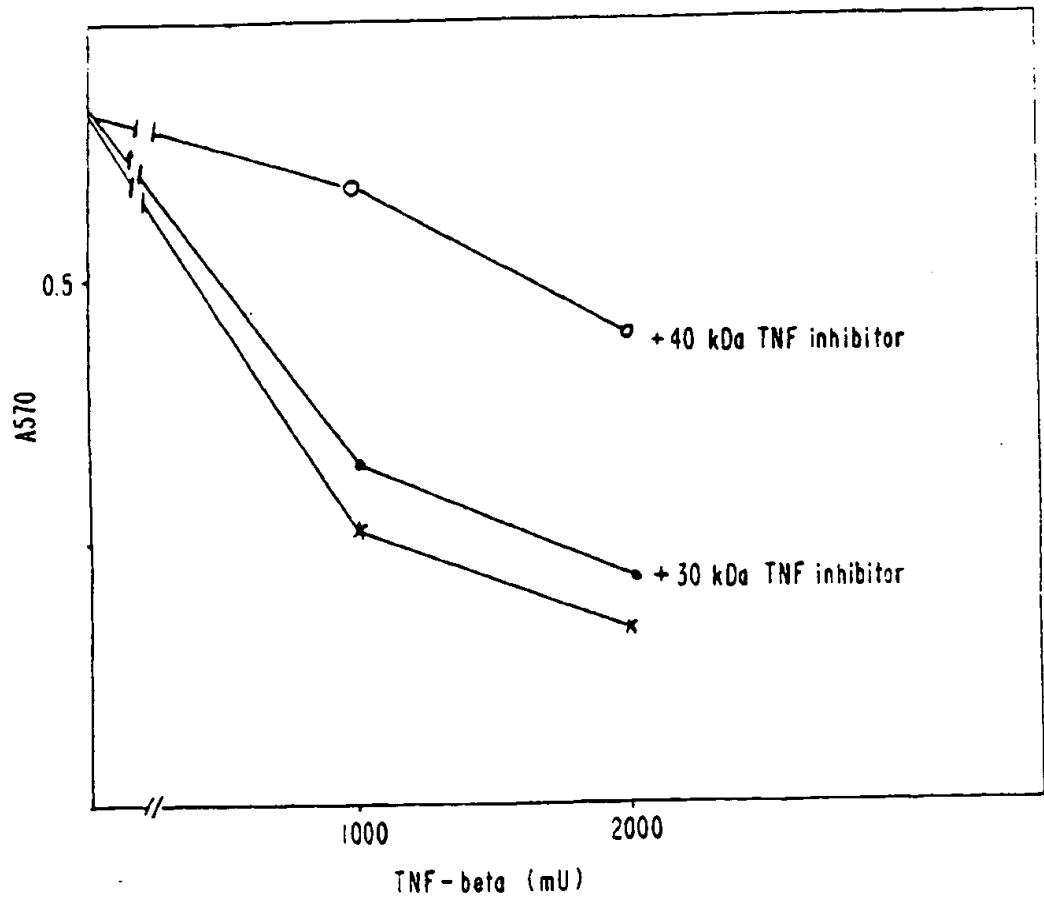


FIG. 41

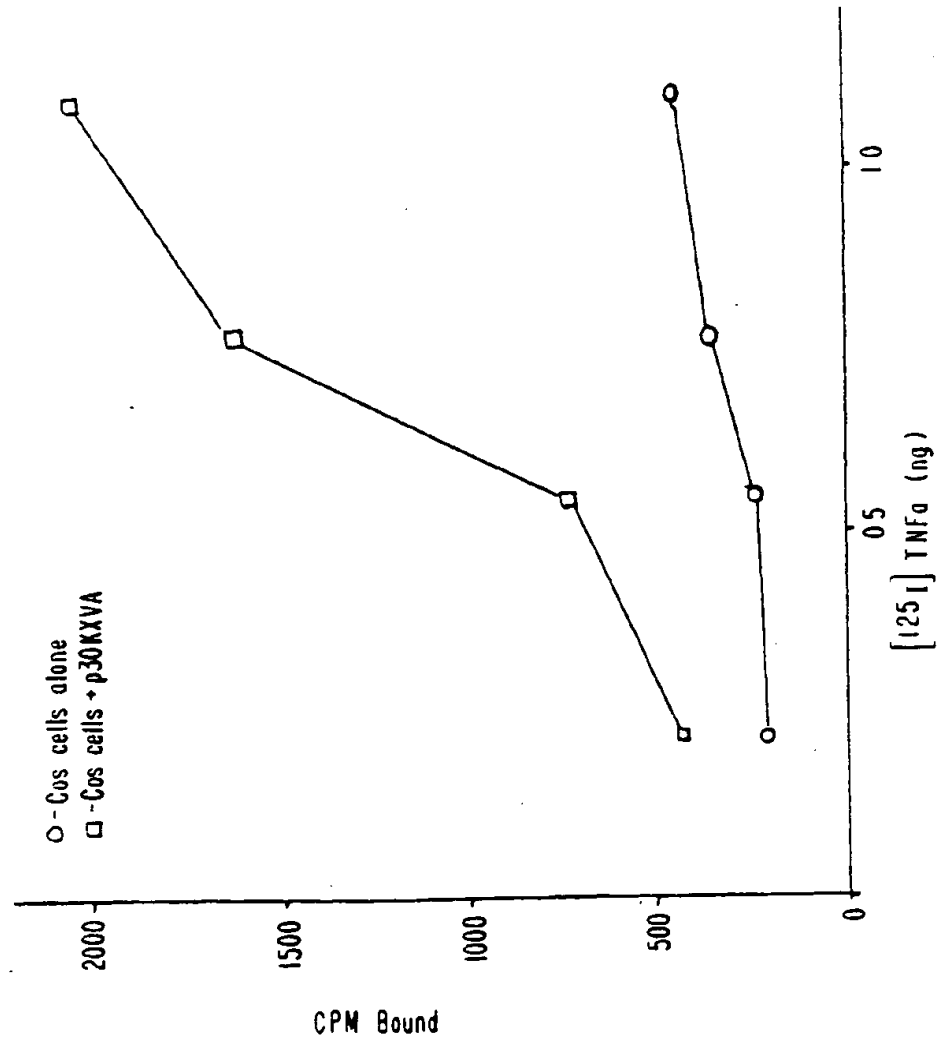


FIG. 42

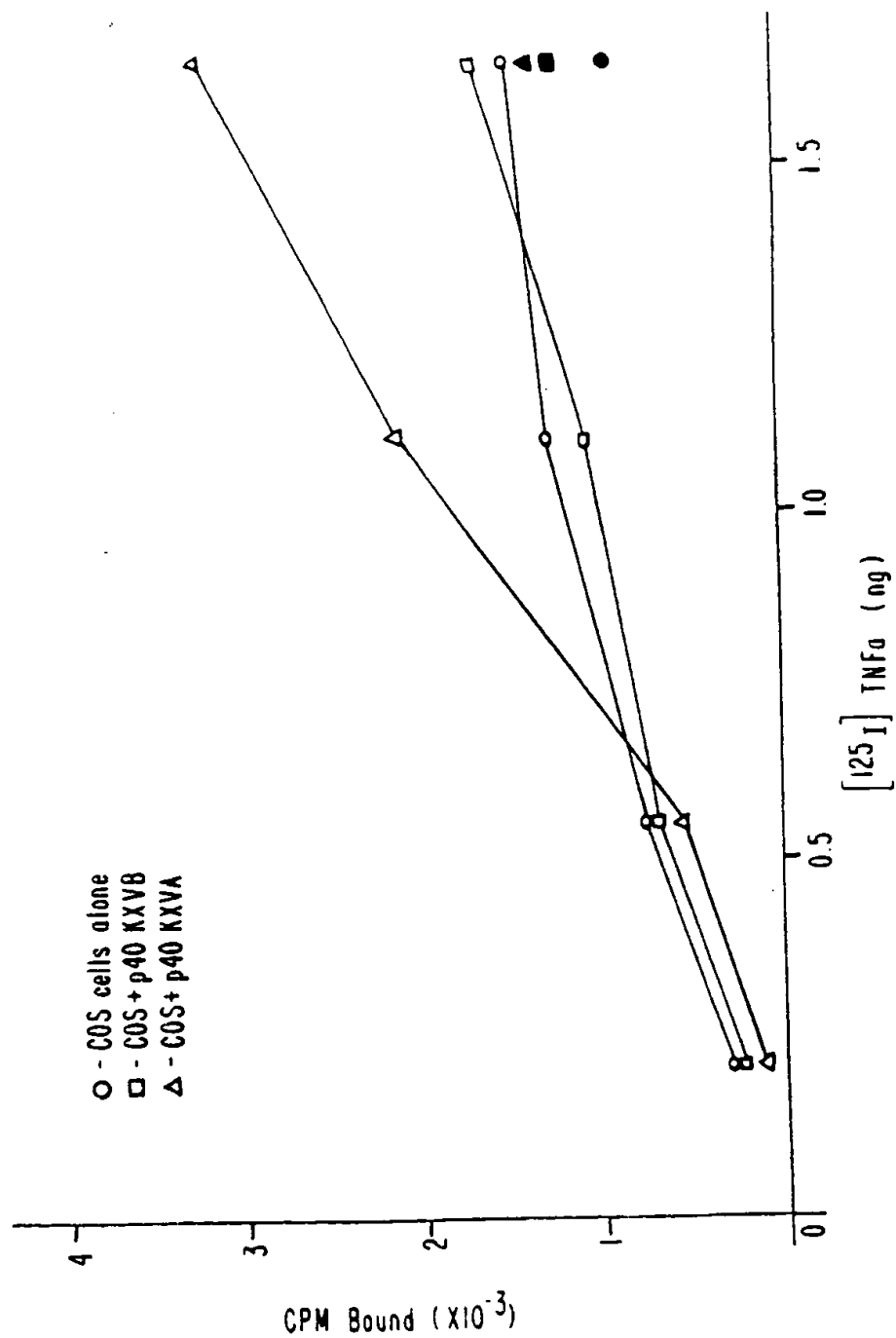


FIG.43

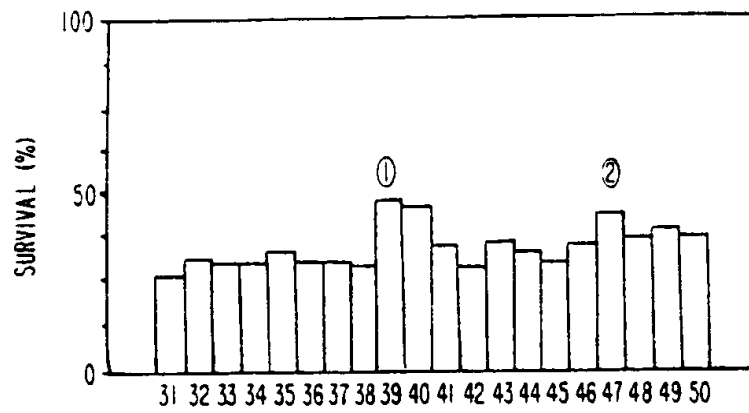


FIG.44A

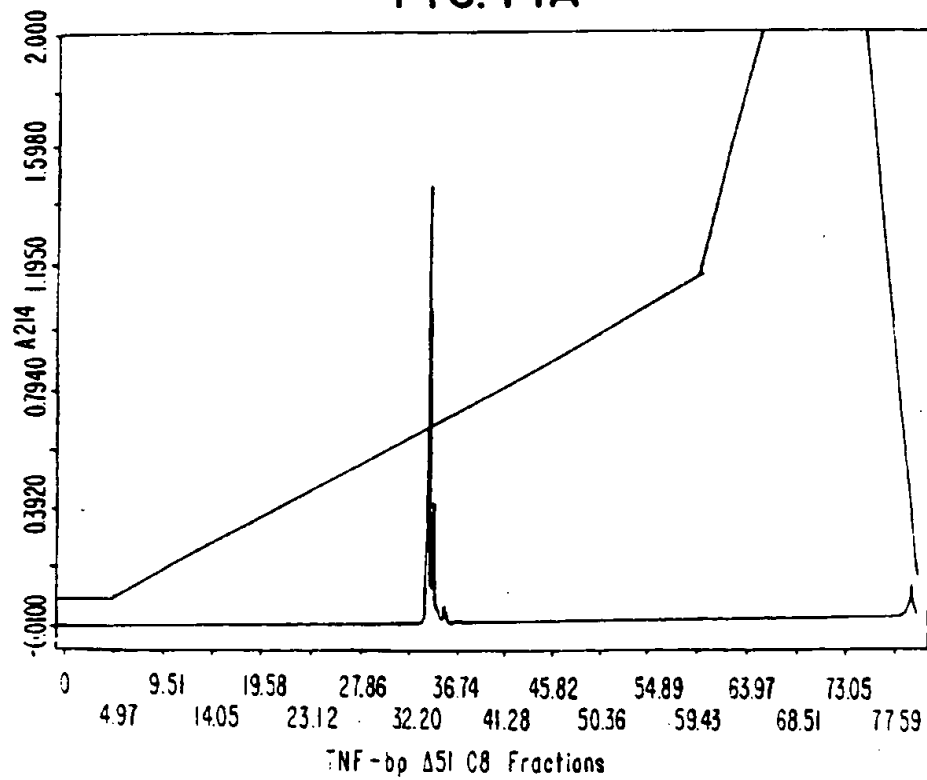


FIG.44B

32 33 34 35 36 37 38 39 40 - TNF $\gamma$  LMW

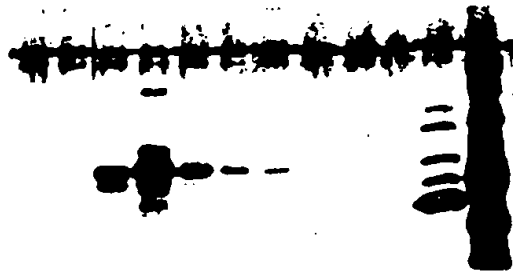


FIG.44C

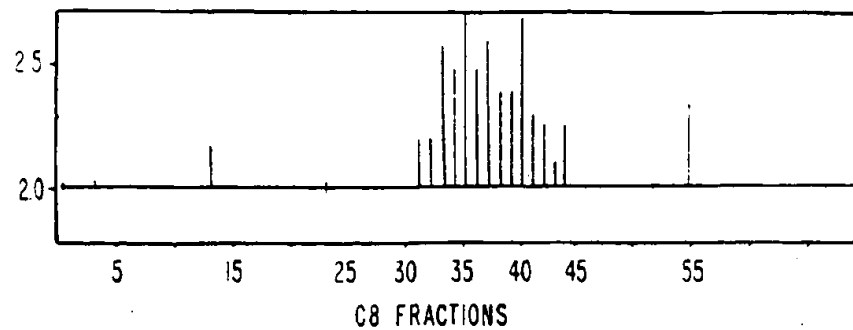


FIG.45A

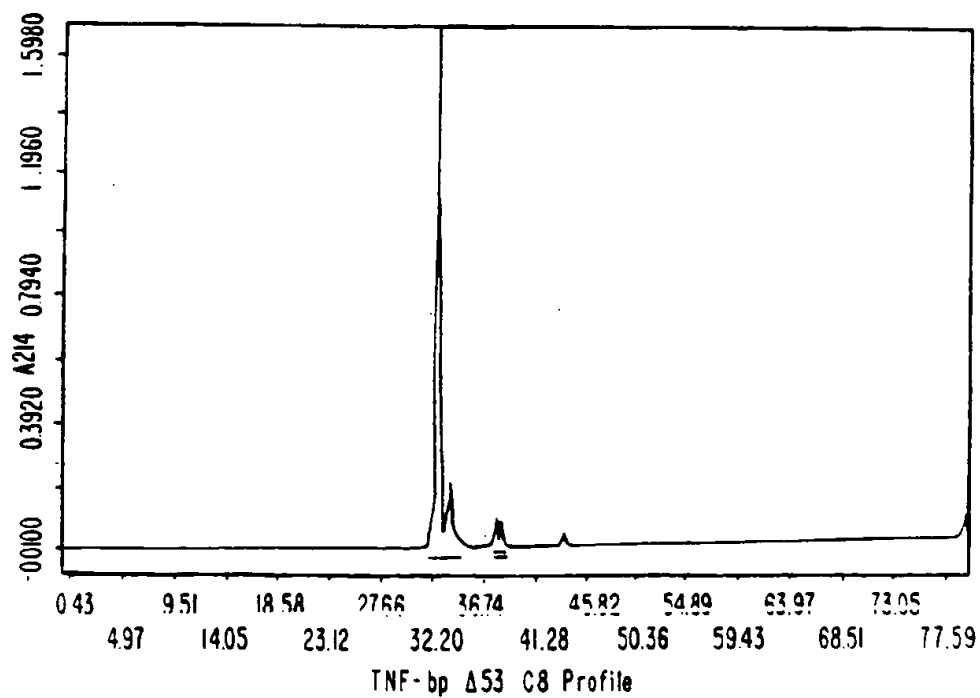


FIG.45B

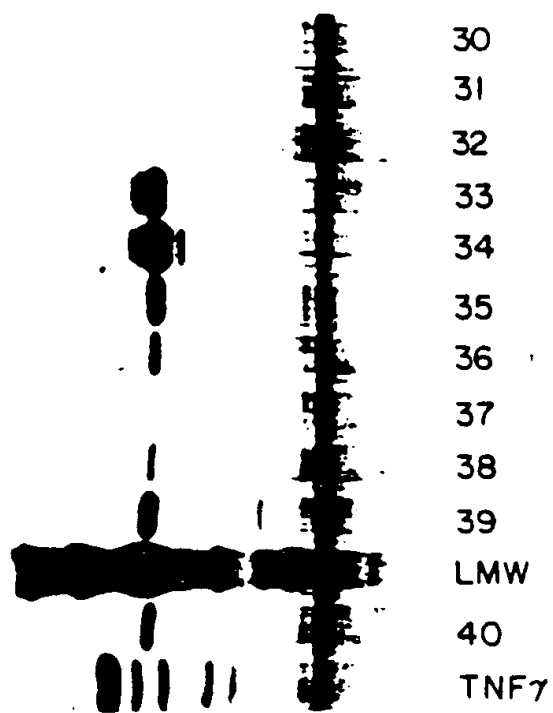
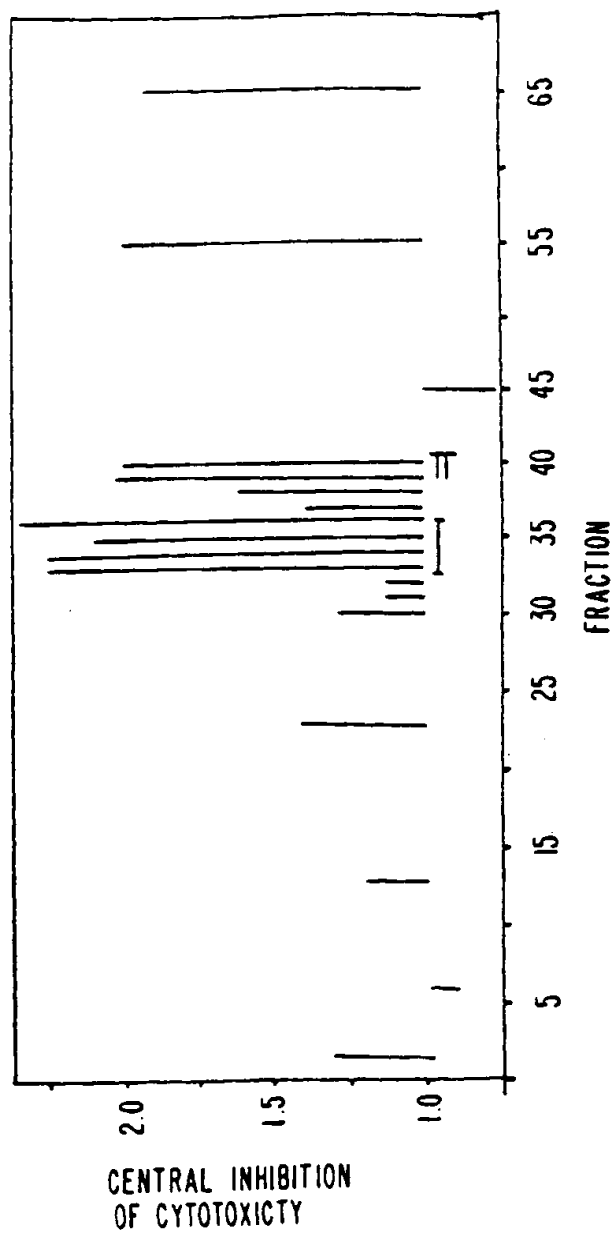




FIG.45C



**THIS PAGE BLANK (USPTO)**



EPA/EPO/OEB  
D-80298 München  
089/2399-0  
TX 523 656 epmu d  
FAX 089/2399-4465

Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets

Generaldirektion 2

Directorate General 2

Direction Générale 2

Grünecker, Kinkeldey,  
Stockmair & Schwanhäusser  
Anwaltssozietät  
Maximilianstrasse 58  
80538 München  
ALLEMAGNE

GRÜNECKER, KINKELDEY, STOCKMAIR  
& SCHWANHÄUSSER  
ANWALTSSOZIELTÄT  
23. Jan. 1998  
FRIST  
TERM  
EINGANG-RECEIVED

Datum/Date

28.01.98

|   |  |
|---|--|
| Zeichen/Ref./Réf.<br>EP 11513 -60/ej  | Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°.<br>90113673.9-2105/0422339 |
| Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire<br>AMGEN BOULDER INC. |  |

TRANSMISSION OF THE CERTIFICATE FOR A EUROPEAN PATENT  
PURSUANT TO RULE 54 (1) EPC

The certificate for a European patent, with the  
specification annexed thereto, is enclosed herewith.

G. TERNIEDEN  
Formalities Officer  
Tel.No.: (+49-89) 2399-4440



**THIS PAGE BLANK (USPTO)**



Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets

# Urkunde      Certificate      Certificat

Es wird hiermit bescheinigt, daß für die in der beigefügten Patentschrift beschriebene Erfindung ein europäisches Patent für die in der Patentschrift bezeichneten Vertragsstaaten erteilt worden ist.

It is hereby certified that a European patent has been granted in respect of the invention described in the annexed patent specification for the Contracting States designated in the specification.

Il est certifié qu'un brevet européen a été délivré pour l'invention décrite dans le fascicule de brevet ci-joint, pour les Etats contractants désignés dans le fascicule de brevet.

Europäisches Patent Nr.

European Patent No.

Brevet européen n°

0422339

Patentinhaber

Proprietor of the Patent

Titulaire du brevet

AMGEN BOULDER INC.  
1885 33rd Street  
Boulder, Colorado 80301/US

München, den  
Munich,  
Fait à Munich, le

28.01.98

Ingo Kober

Präsident des Europäischen Patentamts  
President of the European Patent Office  
Président de l'Office européen des brevets

**THIS PAGE BLANK (USPTO)**